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Hormonal control of membrane transporters in male reproductive tract

Raquel L. Bernardino

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Raquel Alexandra Lages Bernardino

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Hormonal control of membrane transporters in male reproductive tract

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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List of Publications

Publications included in this thesis:

1- **Raquel L. Bernardino**, et al., Pedro F. Oliveira. pH and male fertility: making sense on pH homeodynamics throughout the male reproductive tract. *Submitted*

2- **Raquel L. Bernardino**, et. al., Pedro F. Oliveira. Carbonic anhydrases are involved in mitochondrial biogenesis and control the production of lactate by human Sertoli cells. *Submitted*

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5- **Raquel L. Bernardino**, et. al., Pedro F. Oliveira. (2018) Evaluation of the purity of Sertoli cell primary cultures. In: Alves MG, Oliveira PF (eds) Methods in Molecular Biology, Springer, vol 1. 1748:9-15.

6- **Raquel L. Bernardino**, et. al., Pedro F. Oliveira. (2018) Establishment of Primary culture of Sertoli Cells. In: Alves MG, Oliveira PF (eds) Methods in Molecular Biology, Springer, vol 1. 1748:1-8.

7- **Raquel L. Bernardino**. (2017) Formation and biochemistry of seminal plasma and male accessory fluids. In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE, pp. 207-229. ISBN: 978-1-68108-501-2.

8- **Raquel L. Bernardino**, et. al., Giuseppe Calamita (2016) “Hepatocyte and Sertoli cell Aquaporins, recent advances and research trends”. International Journal of Molecular Science. 17(7): pii: E1096.

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10- **Raquel L. Bernardino**, et. al., Pedro F. Oliveira. (2016) Expression of estrogen receptors alpha (ER-α), beta (ER-β) and G protein-coupled receptor 30 (GPR30) in testicular tissue of men with Klinefelter syndrome. Hormone and Metabolic Research. 48(6):413-5.

11- Meneses MJ, **Raquel L. Bernardino**, et al., Marco G. Alves. (2015) Regulation of Testicular Glucose Metabolism in (Pre)diabetes and its implications to male reproductive health. In: Advances in Medicine and Biology. Volume 89. Nova Science Publishers, Inc, New York, USA, ISBN: 978-1-63483-478-0.

Abstract

Infertility and subfertility affect a significant proportion of couples worldwide. Infertility associated with male factor represents a high percentage of those cases. The establishment of an adequate ionic and metabolic environment throughout the male reproductive tract is crucial for spermatogenesis and hence the production of competent spermatozoa. Spermatogenesis is a complex event that occurs in the testes and depends on the delivery of oxygen, nutrients and hormones into the testicular interstitial fluid. All these processes are dependent on numerous factors, which act in cascade, and anatomical, physiological, hormonal or electrolytic abnormality affect the male reproductive potential. In fact, ionic content of luminal fluids of the male reproductive tract is essential for the normal development of spermatozoa. Among the several ions, HCO_3^- and H^+ have a crucial role, mainly due to their intervention on pH homeostasis. As hormonal dysregulation is frequently associated with male infertility and the content of fluids surrounding spermatozoa is so important for the establishment of the male reproductive potential, it is of major relevance to deepen the knowledge on the possible interconnection between hormonal dysregulation and ion transport in the male reproductive tract. Hence, the first objective of this work was to analyse the importance of HCO_3^- on human Sertoli cells (hSCs). The testicular somatic Sertoli cells (SCs) are responsible for production/control of the fluid that surrounds all germ cells during spermatogenesis known as the seminiferous tubular fluid (STF). By the specific inhibition of carbonic anhydrases (CAs) in hSCs, and consequently decreased production of HCO_3^-, we observed a decreased expression of markers for mitochondrial biogenesis. This was followed by alterations in the metabolism of these cells characterized by an increased production of lactate and alanine. Inhibition of CAs also caused changes in hSCs lipid metabolism. Our results suggest that CAs are also essential for the expression of genes involved in hSCs glucose and lipid metabolism. Thus, CAs can play a key role in spermatogenesis by controlling lactate production and by modulating the expression of genes associated with mitochondrial biogenesis.

As HCO_3^- is important for SCs metabolism and physiology, and SCs are responsible for the production of the STF, we hypothesized that the establishment of the seminiferous intratubular environment may be compromised by alterations in the production and transport of HCO_3^- by hSCs. The second objective of this work was to elucidate the mechanisms involved in the membrane transport of HCO_3^- in hSCs. We further aimed to evaluate the impact of hormonal dysregulation, specifically how high doses of estrogen (E2) influence the expression and functionality of those transport mechanisms. In hSCs exposed to elevated doses of E2 we observed an increased intracellular pH (pHi). This alteration in the pHi is due to an increased concentration of HCO_3^- in the intracellular compartment caused by an increased protein

expression of HCO_3^- transporters. E2 also changed the transcellular transport through hSCs. These alterations caused by E2 on SCs may be responsible for a dysregulation in STF homeostasis and consequently the arrest of spermatogenesis and sperm production.

Spermatogenesis development occurs in a controlled environment attained by a well-established STF, prompted by the existence of the blood-testis barrier (BTB), also known as SC barrier. The controlled composition of this fluid is also due to the movement of water and several solutes. Among those, glycerol has been reported as a key player for the normal function of the BTB and SCs. High concentrations of glycerol are responsible for alterations in STF homeodynamics, due to changes in BTB permeability that result in infertility. Thus, the third objective of this work was to determine the molecular mechanisms responsible for glycerol transport in the BTB. We further evaluated the effect of high E2 levels on glycerol permeability, which is mediated by aquaglyceroporins (AQPs), in mouse SCs (mSCs). We observed that high E2 levels decreased mSCs permeability to glycerol, while downregulating AQP9 expression. These results highlight that E2 is an important regulator of mSCs physiology and secretion through changes in AQP9 expression and function. Thus, alterations in mSCs permeability to glycerol induced by E2 may be the cause for male infertility in cases associated with the presence of high levels of E2. One last aim of this work was to highlight an eventual correlation of our findings in SCs with a clinical condition related to high levels of E2 and male infertility. We started by performing an evaluation of the expression of estrogen receptors in the testicular tissue of men with a genetic syndrome associated with hyperestrogenism, the Klinefelter syndrome. We observed that the abundance of GPR30 in individuals with Klinefelter syndrome is significantly higher relative to individuals with a normal genotype. This increase may have a direct impact on testicular development and physiology and, since GPR30 is essential to mediate the effects of estrogen in steroidogenesis, our data illustrate that GPR30 may underpin some of the testicular alterations observed in men with Klinefelter syndrome.

In conclusion, we describe here for the first time key molecular mechanisms responsible for the production and transport of HCO_3^-, H^+ and glycerol in the testis, particularly in SCs. Our study also showed that E2 is able to modulate the homeodynamics of those ions and molecules in hSCs, and that alterations in these components in STF may result in deleterious consequences in spermatogenesis and sperm production. However, there is still much to be unveiled concerning the role of those membrane transporters in male fertility, and particularly on the influence of E2 on all those events. It would be of great interest to evaluate the expression and function of membrane transporters in SCs and testicular tissue from individuals suffering from pathological conditions associated with increased levels of estrogens (i.e., Klinefelter syndrome, idiopathic infertility...), in which we have already detected a dysregulation in the expression of estrogen receptors.

Resumo

A infertilidade e subfertilidade afetam um elevado número de casais em todo o mundo. O fator masculino contribui com uma grande percentagem para os casos de infertilidade descritos. Estabelecer um ambiente adequado em todo o trato reprodutivo masculino é crucial para todo o processo de espermatogénese e para a produção de espermatozoides competentes. De facto, a espermatogénese é um processo complexo que ocorre no testículo sendo dependente da existência de oxigénio, nutrientes e hormonas no fluido intersticial. Todos os processos que ocorrem durante a espermatogénese são dependentes de inúmeros fatores, que atuam em cascata, e qualquer anomalia anatómica, fisiológica, hormonal ou eletrolítica pode alterar o potencial reprodutor masculino. De fato, o conteúdo iónico dos fluidos luminais do trato reprodutor masculino é essencial para o desenvolvimento normal dos espermatozoides. Entre os vários iões, o HCO_3^- e o H^+ têm um papel crucial, principalmente devido ao seu papel na homeostase do pH.

Visto que a desregulação hormonal está frequentemente associada à infertilidade masculina e o conteúdo de fluidos que banham os espermatozoides é tão importante para o estabelecimento do potencial reprodutivo, é importante aprofundar o conhecimento sobre a possível interconexão entre desregulação hormonal e transporte iónico no trato reprodutor masculino. Consequentemente, o primeiro objetivo deste trabalho foi analisar a importância do HCO_3^- nas células de Sertoli humanas (hSCs). As células de Sertoli (SCs) são as principais responsáveis pela produção/controle do fluido tubular seminífero (STF), o fluido que envolve todas as células germinativas durante a espermatogénese. Ao inibirmos especificamente as anidrases carbónicas (CAs) em hSCs e, consequentemente, diminuir a produção de HCO_3^-, observamos uma diminuição da expressão de marcadores da biogénese mitocondrial. Estes resultados foram seguidos ainda por alterações no metabolismo das células, com um aumento da produção de lactato e alanina. A inibição das CAs também causou alterações no metabolismo lipídico das hSCs. Assim, os nossos resultados sugerem que as CAs são essenciais para a expressão de genes envolvidos no metabolismo da glucose e dos lípidos nas hSCs. Nestas células, as CAs desempenham um papel fundamental na espermatogénese, controlando a produção de lactato e modulando a expressão de genes associados à biogénese mitocondrial.

Uma vez que o HCO_3^- é importante para o metabolismo e a fisiologia das SCs, e estas células são responsáveis pela produção do STF, colocamos como hipótese que o estabelecimento do ambiente intratubular seminífero pode estar comprometido caso ocorram alterações na produção e transporte de HCO_3^- por hSCs. Assim, o segundo objetivo deste trabalho foi o de elucidar os mecanismos envolvidos no transporte membranar de HCO_3^- em hSCs. Tivemos também como objetivo determinar o impacto da desregulação hormonal, especificamente o

de altas doses de estrogénio (E2), na expressão e funcionalidade desses mecanismos de transporte. Nas hSCs expostas a doses elevadas de E2 observamos um aumento do pH intracelular (pHi). Esta alteração no pH deveu-se ao aumento da concentração de HCO_3^- no compartimento intracelular, como resultado do aumento da expressão proteica de alguns transportadores de HCO_3^-. A exposição a doses elevadas de E2 também provocou alterações no transporte transcelular nas hSCs. Essas alterações causam desregulação na produção de STF e consequentemente na espermatogénese e produção dos espermatozoides.

O desenvolvimento da espermatogénese ocorre num ambiente controlado sendo o STF, criado pela existência da barreira hemato-testicular (BTB) também designada por barreira das SCs, essencial nesse processo. Concentrações elevadas de glicerol são responsáveis por alterações na homeodinâmica do fluido tubular seminífero devido a alterações na permeabilidade da BTB, que consequentemente leva a infertilidade. Assim, o terceiro objetivo deste trabalho foi o de determinar os mecanismos moleculares responsáveis pelo transporte de glicerol na BTB. Nós avaliámos ainda o efeito do E2 na permeabilidade do glicerol, que se sabe ser mediado pelas aquagliceroporinas (AQPs), em SCs de ratinho (mSCs),. Observamos que níveis elevados de E2 diminuíram a permeabilidade das mSCs ao glicerol, diminuindo também a expressão da AQP9. Assim, o E2 é um importante regulador da fisiologia e secreção das mSCs através de alterações induzidas na expressão e função da AQP9. Assim, alterações na permeabilidade ao glicerol induzidas por E2 nas SCs podem ser a causa de infertilidade masculina em casos associados à presença de elevados níveis de E2.

Um último objetivo deste trabalho foi o de permitir uma eventual correlação dos nossos resultados em SCs com uma condição clínica relacionada com elevados níveis de E2 e infertilidade. Assim, o nosso trabalho começou com uma avaliação da expressão de recetores de estrogénio no testículo de homens com uma síndrome genética associada ao hiperestrogenismo, a síndrome de Klinefelter. Observámos que a abundância de GPR30 em homens com síndrome de Klinefelter está aumentada. Este aumento pode ter um impacto direto no desenvolvimento e fisiologia testicular e, como o GPR30 é essencial para mediar os efeitos do estrogénio na esteroidogénese, os nossos resultados demonstram que o GPR30 pode sustentar algumas das alterações testiculares observadas em homens com síndrome de Klinefelter.

Concluindo, descrevemos pela primeira vez os principais mecanismos moleculares responsáveis pela produção e transporte testicular de HCO_3^-, H^+ e glicerol, particularmente em SCs. O nosso estudo mostrou também que o E2 é capaz de modular a homeodinâmica destes iões e moléculas em hSCs, sendo que alterações na dinâmica desses componentes do STF podem resultar em consequências nocivas para a espermatogénese e para a produção de espermatozoides. No entanto, ainda há muito a ser elucidado sobre o papel

destes transportadores de membrana na fertilidade masculina e, particularmente, sobre a influência de E2 em todos esses processos. Seria de grande interesse avaliar a expressão e função de transportadores de membrana em SCs e tecido testicular de indivíduos que sofrem de condições patológicas associadas a níveis aumentados de estrogénios (p.e. síndrome de Klinefelter, infertilidade idiopática ...), em que já demonstramos uma desregulação na expressão de recetores de estrogénio.

Table of Contents

List of Abbreviations

^{1}H-NMR	Proton nuclear magnetic resonance
5α-DHT	5α-dihydrotestosterone
ACC	Acetyl-CoA carboxylase
ACSL1	Acyl-CoA synthetase long chain family member 1
ACT	Acetazolamide
AE	Anion exchanger
ALT	Alanine aminotransferase
AMRC	Apical mitochondria-rich cells
AP	Alkaline phosphatase
AQPs	Aquaporins
BCECF-AM	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester
BEB	Blood-epididymal barrier
BSA	Bovine serum albumin
BTB	Blood-testis barrier
CaM	Calmodulin
CAs	Carbonic anhydrases
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CLD	Congenital chloride diarrhoea
CTR	Control
DAB	3,3'-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
E2	17β-estradiol
ERα	Estrogen receptors alpha
ERβ	Estrogen receptors beta
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GH	Growth hormone
GLUTs	Glucose transporters
GnRH	Gonadotropin releasing hormone
GPR30	G protein-coupled estrogen receptor
HBSS	Hank´s balanced salt solution

HiF1-α	Hypoxia-inducible factor 1-alpha
HPT	Hypothalamus–pituitary–testis
hSCs	Human Sertoli cells
HSL	Hormone sensitive lipase
IGF-1	Insulin-like growth factor 1
ITS	Insulin-Transferrin-Selenium
JC-1	1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide
KO	Knouckout
KS	Klinefelter syndrome
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LPA	Lysophosphatidic acid
MCTs	Monocarboxylate transporters
mSCs	Mouse Sertoli cells
mtDNA	Mitochondrial DNA
MT-ND1	NADH dehydrogenase 1
NBCe	Electrogenic Na^+/HCO_3^- cotransporters
NBCn	Electroneutral Na^+/HCO_3^- cotransporters
NCBTs	Na^+-coupled HCO_3^- transporters
NDCBE	Na^+-driven Cl^-/HCO_3^- exchanger
NHEs	Na^+/H^+ exchanger
NRF-1	Nuclear respiratory factor-1
PAP	Prostatic acid phosphatase
PBS	Phosphate-buffered saline solution
PDH	Pyruvate dehydrogenase
PFK	Phosphofrutokinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
pHi	Intracellular pH
pHo	Extracellular pH
PKA	Protein kinase A
PLIN2	Perilipin 2
qPCR	Quantitative reverse transcriptase polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
sAC	Soluble adenylyl cyclase
SCs	Sertoli cells
SFLS	Stopped flow light scattering

SIRT1	Sirtuin 1
SLC	Solute carrier
SPM	Sperm preparation medium
STF	Seminiferous tubular fluid
T3	Tri-iodothyronine
T4	Thyroxine
TCA	Tricarboxylic acid
TRP	Transient receptor potential

Chapter 1

Introduction

This chapter was adapted from the published/ submitted works:

Maria J. Meneses, ***Raquel L. Bernardino****, et al., Marco G. Alves. (2015) Regulation of Testicular Glucose Metabolism in (Pre)diabetes and its implications to male reproductive health. In: Advances in Medicine and Biology. Volume 89. Nova Science Publishers, Inc, New York, USA, ISBN: 978-1-63483-478-0.*

Raquel L. Bernardino*, et al., Pedro F. Oliveira. pH and male fertility: making sense on pH homeodynamics throughout the male reproductive tract. Submitted*

Raquel L. Bernardino*, et al., Giuseppe Calamita. Hepatocyte and Sertoli cell aquaporins, recent advances and research trends. International Journal of Molecular Sciences. 17(7):1096.*

Raquel L. Bernardino*. (2017) Formation and biochemistry of seminal plasma and male accessory fluids. In: Alves MG, Oliveira PF (eds) Biochemistry of Andrology, vol 1. Andrology: Current and Future Developments. Bentham Science Publishers, Sharjah, UAE,pp. 207-229. ISBN: 978-1-68108-501-2.*

General aspects

Testicular Physiology

The establishment of male fertility is a complex process that requires specific and controlled interactions between different tissues and cells of the male reproductive tract and accessory glands. The male reproductive tract is formed of heterogeneous tissues, including the testis, efferent ducts, epididymis and vas deferens. The mammalian testis is a complex organ, coated for a capsule constituted by complex fibrous structure in various distinct tissue layers. The major component of the capsule, the tunica albuginea, is distinguished by the existence of fibroblasts interspersed in collagen fibers [1], from which septations extend toward the testicular mediastinum, separating the human testis into 200–300 lobules [2] (Figure 1.1). Tunica albuginea has several physiological functions in male reproduction, including preservation of the interstitial pressure within the testis, boost the transport of sperm out of the testis into the epididymis and control of the blood flow through the testis [1].

Each testicular lobule encloses coiled seminiferous tubules (Figure 1.1). Within the testis two compartments are formed, the interstitial and tubular, that present different cell types and fluid flow [3]. The interstitial compartment is the space outside the seminiferous tubules. Here, the most important cells are the Leydig cells that actively produce and secrete testosterone, the main male sexual hormone [4, 5] (Figure 1.1). In humans, the interstitial compartment represents about 12–15% of the total testicular volume, 10–20% of which is composed of Leydig cells. Human testes have approximately 200×10^6 Leydig cells [3]. The proliferation rate of these cells in the adult testis is low and influenced by luteinizing hormone (LH). In addition to Leydig cells, the interstitial compartment contains macrophages, lymphocytes, mesenchymal fibroblasts, extracellular matrix, and small blood capillaries [4]. The tubular compartment represents about 60-80% of the total testicular volume. The seminiferous tubules are avascular and with no nerves penetrating through their walls [6]. Overall, the human testis contains about 600 seminiferous tubules. The lobules filled by seminiferous tubules are separated by extensions of the tunica albuginea that open on both ends into the rete testis (Figure 1.1). Of note, all the processes involved in the production of male gametes (spermatogenesis), occur within the seminiferous tubules [3]. These tubules end at the rete testis, which is a network of tubules that empties into the efferent ductules. Spermatozoa are transported through the efferent ductules into the epididymis, and then enter the vas deferens, which through peristalsis propels them to the ejaculatory duct [2].

The tubular compartment contains the germ cells and two different types of somatic cells, the Sertoli (SCs) and peritubular cells [2]. The seminiferous tubules are sheltered by a lamina,

which consists of a basal membrane, a layer of collagen and the peritubular cells (myofibroblastic cells). These cells are stratified and form concentric layers that are separated by collagen layers [7]. The peritubular cells have an essential role in the transport of sperm [8, 9], and contribute to several testicular functions by secreting factors involved in cellular contractility (panactin, desmin, gelsolin, smooth muscle myosin and actin), components of the extracellular matrix (collagen, laminin, vimentin, fibronectin, growth factors, fibroblast protein) and adhesion molecules [7, 10-13].

The SCs extend from the base to the apex of the epithelium in a direct interaction with the developing germ cells [14] (Figure 1.1). About 35–40% of the volume of the germinal epithelium is comprised by these cells. The human testis of reproductive age contains about 800–1200×10^6 SCs [15]. These somatic cells are commonly referred as “nurse cells”, because they are responsible for providing nutritional and energy support to developing germ cells, and for creating an immunologically protected space for the development of germ cells [16]. Adjacent SCs form tight junctions with each other in such a way that nothing larger than 1kDa can pass from the outside to the inside of the tubule. The tight junctions formed between adjacent SCs create the Sertoli/blood–testis barrier (BTB) that physically divides the seminiferous epithelium into basal and apical compartments (Figure 1.1). This barrier regulates the movement of substances, such as nutrients and wastes, in and out of the seminiferous epithelium [17, 18]. The cytoskeleton of SCs is responsible for the organization of the seminiferous epithelium and plays a fundamental role in facilitating germ cells movement. In addition to the tight junctions, other types of associations occur between the SCs to strengthen the BTB, such as adherens junctions (eg: ectoplasmic specialization, a testis-specific adherens junction type) and intermediate filament-based desmosome-like junctions [14, 16, 19]. The relevance of these junctions goes far beyond the physical and nutritional support as they must undergo extensive restructuring during germ cell migration on the course of spermatogenesis.

Several proteins, products and factors are known to be secreted by SCs, including: proteases, protease inhibitors, hormones, energy substrates, growth factors, paracrine factors, inhibin, transferrin, androgen-binding protein, plasminogen activator, glycoproteins, sulpho-proteins, myo-inositol and other extracellular matrix components [20-26]. Notably, the number of SCs is also an important determinant of testis size, and this number is directly related to the number of germ cells [20]. Germ cells are dependent on SCs not only for structural support, but also for nutritional and energetic support. The SCs convert glucose to lactate that is known to be used as substrate and to influence the survival of germ cells. Although glucose is one of the most used substrates by SCs, they can also metabolize other substrates such as ketone bodies and fatty acids [16, 20]. These processes are vital for the production and export of lactate that can be conditioned by several factors. For instance, it has been recently reported

that insulin-deprived SCs altered the expression of metabolism-associated genes implicated in the export and production of lactate, as well as the consumption of glucose and secretion of lactate [27]. The SCs also control the composition of the seminiferous tubular fluid (STF) and the physicochemical milieu where spermatogenesis occurs. The STF serves as a mean of transport of sperm, and also helps to maintain a proper microenvironment required for normal spermatogenesis [28, 29].

Spermatogenesis

Spermatogenesis is a complex biological process that produces spermatozoa in the seminiferous tubules of the mammalian testis. It involves an important balance between self-renewal and differentiation of spermatogonial stem cells to ensure an endless production and release of mature spermatozoa [30]. A fertile man produces more than 40 million spermatozoa per day, beginning at puberty and spanning his entire reproductive life [31]. The duration of the complete spermatogenic process varies according to species and in human it spans 74–76 days [30]. The SCs are responsible for the movement of germ cells from the base of the tubule toward the lumen and for the release of mature spermatozoa into the lumen. Moreover, as discussed above, the BTB, composed by SCs, divides the epithelium into two compartments: the basal compartment, in which spermatogonia, preleptotene, and leptotene spermatocytes exist; and the adluminal compartment, in which meiotic spermatocytes and spermatids in different stages reside [32, 33] (Figure 1.1). Spermatogenesis is controlled by several factors, (endocrine, paracrine and autocrine). Spermatogenesis can be divided into four different phases that include: mitosis, meiosis, spermiogenesis and spermiation. In the early steps of spermatogenesis, diploid spermatogonia (2n) proliferate and generate two different populations of cells: one subpopulation of stem cells identical to their progenitors; other subpopulation, the majority, starts a differentiation process and differentiate into spermatozoa. In this process there is a subpopulation of spermatozoa and germ cells in different stages that undergo apoptosis [34-37]. This phase of spermatogenesis is of great complexity and requires a time-specific period for completion depending of the species. For instance, it requires 6–9 weeks for completion in human. Spermatogonia are the most primitive diploid germ cells (2n) that divide by mitosis and reside on the basement membrane of seminiferous epithelium (Figure 1.1). In the mitotic phase, the spermatogonia are self-renewed or undergo differentiation, with both cases involving several mitotic divisions. Little is known about the division of spermatogonia in humans, but four different types of spermatogonia have been identified in human: type A Dark, type A Pale, type A Long and type B [34, 36-38]. The main morphological features used to distinguish between these four types of spermatogonia were the shape and staining characteristics of the nucleus, the position of the nucleolus, the

structure of mitochondrial cristae, the association of the endoplasmatic reticulum with mitochondria, the presence or absence of glycogen in the cytoplasm of the cells and the presence of the filamentous structures of cytoplasm [38]. The Type A Long and A Dark spermatogonia are suggested to be the least morphologically differentiated and are considered A Dark the subpopulation of stem cells. Each spermatogonium is in touch with the basal lamina in the seminiferous tubule. Notably, this proximity progressively decreases with the degree of spermatogonia differentiation. The type A Long spermatogonia are the flat cells lying parallel to the basal lamina, through the type A Dark, type A pale and type B [33, 38]. Type B is the most differentiated type of spermatogonia since this differentiation only occurs in two thirds of the type A pale spermatogonia. Type B spermatogonia enter into meiosis and differentiated into preleptotene spermatocytes which in turn differentiate into zygotene spermatocytes. The BTB separates these two states of spermatocytes, the preleptotene spermatocytes located in the basal compartment and the pachytene spermatocytes in adluminal compartment [14, 31].
Meiosis is the second phase of spermatogenesis and begins in the basal compartment with prophase and ending at the adluminal compartment [39]. Primary spermatocytes go into the first meiotic division and form leptotene, zygotene, pachytene and diplotene spermatid. During these phases the chromosomes condense, form pairs of homologous chromosomes, synapses are completed and then substituted by crossing-over and homologous recombination. In diplotene stage, chromosomes are unsynapsed and the cell divides. Meiosis is characterized by the separation of chromosomes that occurs during the metaphase, anaphase and telophase of the first meiotic division, after which secondary spermatocytes are originated [40, 41] (Figure 1.1). The prophase of the first meiosis lasts 1–3 weeks, whereas the other phases of the first meiosis and the entire second meiosis are completed within 1–2 days in man [3]. Secondary spermatocytes are formed from the first meiotic division and contain haploid chromosomes in duplicate. During the second meiotic division secondary spermatocytes originate four haploid spermatids (n) [3, 39]. Spermiogenesis and spermiation are the last phases of spermatogenesis in which the spermatids undergo several alterations. During spermatid development, the nucleus elongates and condenses. The nucleus of spermatids contains compacted DNA following the replacement of nucleosomal histones by transition proteins and subsequently by protamines [42]. Spermatids undergo several transformations leading to the final production of differentiated elongated spermatids and spermatozoa (Figure 1.1). For human, originally 12 spermatid maturation steps were described. These steps include nucleus condensation, the formation of a flagellum and the expulsion of a large part of cytoplasm [3]. The differentiation into the extremely specialized sperm cells is one of the most significant cell developmental processes that occur in biological systems. It involves phases of acrossome development, nuclear elongation and condensation,

the formation of middle piece and tail, and the reduction of cytoplasmatic volume [39]. The SCs play an essential role in reducing the cytoplasmatic volume of the elongated spermatids. The adhesive contacts and ectoplasmic junctional specializations between SCs and spermatids are shattered, thereafter the release of elongated spermatids into the lumen of seminiferous tubule occurs in a process named spermiation [20, 39].

Hormonal Regulation of Spermatogenesis

Spermatogenesis is an intricate process, controlled by several interacting mechanisms that differ according to the consecutive developmental stages: fetal, infantile, pubertal and adult. It is tightly regulated by the hypothalamus–pituitary–testis (HPT) axis. Within this axis, neurons of the hypothalamus produce gonadotropin releasing hormone (GnRH). Regulation of gonadotropin secretion involves a complex interaction between stimulation by GnRH from the hypothalamus and feedback control by sex steroids and inhibin from the testes, besides autocrine/paracrine modulation by other factors within the pituitary [2, 43]. Pulsatile GnRH signals stimulate gonadotropic cells in the anterior pituitary to release follicle-stimulating hormone (FSH) and LH that then act on the testis to regulate the spermatogenic potential [16, 18, 43]. LH is of upmost importance for the stages of immature and mature adult Leydig cells, being the main inducer of these adult cells differentiation and the responsible for the maintenance of its high rates of proliferation [44]. LH acts on Leydig cells through LH receptors present in the surface of these somatic cells of the testis and stimulates the production of testosterone, a steroid hormone that diffuses into the seminiferous tubules. Testosterone is the main secreted product of the testis, the daily production rate being 5–7 mg in men [2]. Interestingly, testosterone plasma levels are strictly correlated to LH levels and it was described that pulsatile LH concentrations stimulate testosterone secretion in a dose-dependent manner [31, 43]. SCs are the only testicular cells that express receptors for testosterone and FSH. FSH is a member of the glycoprotein hormone family which binds to its receptor in SCs and has typically been described to be involved in the beginning of pubertal spermatogenesis [30]. This hormone is crucial to stimulate spermatogenesis, as it maintains testicular size, seminiferous tubular diameter and a suitable number of sperm with normal motility [2, 43]. FSH also regulates DNA synthesis in spermatogonia, spermatogonial proliferation and differentiation [34, 45].

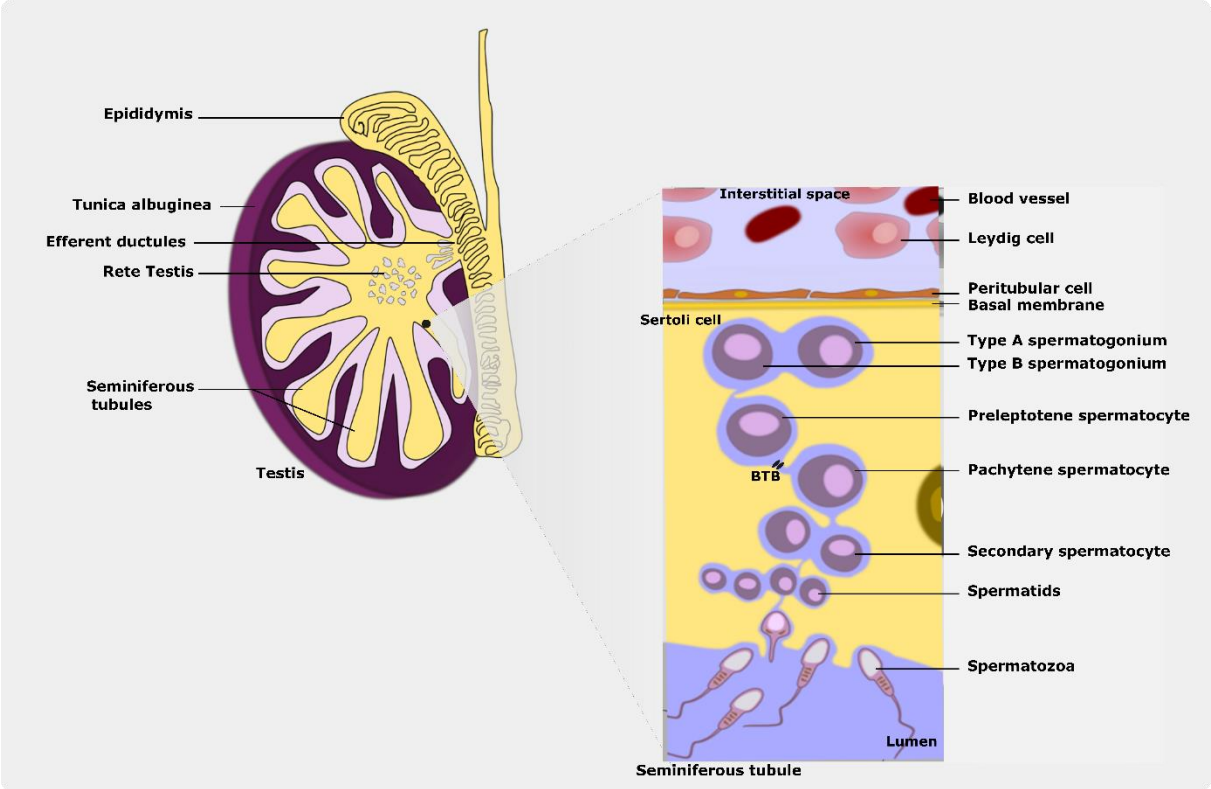


Figure 1.1 Schematic simplified representation of the human testis and spermatogenesis. The testis is coated by tunica albuginea, and is divided in lobules. The seminiferous tubules, where spermatogenesis occurs, are coiled in the testis lobules. The Leydig cells and blood vessels are found in the interstitial space. The seminiferous epithelium is composed of Sertoli cells, which form the Sertoli/blood-testis barrier (BTB), and developing germ cells at different stages. The Sertoli cells adhere to the basal membrane where spermatogonia are also adherent. Type A Spermatogonia divide and develop into type B spermatogonia. The primary spermatocytes (preleptotene and pachytene) start meiosis and divide in secondary spermatocytes, which in turn divided into haploid spermatids that migrate towards the lumen where the fully formed spermatozoa are finally released.

The HPT axis is regulated by feedback mechanisms. The production of inhibin by SCs, as well as testosterone and 17β-estradiol (E2) by Leydig cells lead to a negative feedback that decreases the secretion of GnRH in the hypothalamus and of LH in the pituitary [46]. Sex steroids (e.g. E2) seem to play a slight role in the feedback control of FSH secretion while inhibin is the key factor implicated in the testicular regulation of FSH secretion. Notably, there is an inverse relationship between inhibin's circulating level and FSH ones [2]. In the last years, there has been an increasingly comprehension that sex steroid hormones are involved in several biological mechanisms, including the homeostasis of energy balance and energy metabolism in male reproductive activity [47, 48]. The GnRH pulse is extremely susceptible to energetic deficits, environmental contaminants and intense exercise [49-51]. For instance, Trumble and collaborators [49] have described that fasting causes suppression of GnRH pulses, which consequently causes a decrease in the levels of LH and testosterone production by Leydig cells disturbing male reproductive function.

Androgens are considered the main sex male hormones, namely testosterone, while estrogens are usually referred as the sex female hormones. Nevertheless, androgens and estrogens are present in both sexes. Therefore, sexual distinctions are not qualitative differences, but result from quantitative divergence in hormone concentrations and differential expressions of steroid hormone receptors [52]. In men, most serum estradiol is produced by peripheral aromatization of androgens secreted by Leydig cells. The cytochrome P450 enzyme aromatase is responsible for catalyzing this reaction and is primarily present in adipose tissue but also functions in skin and liver. In the testis aromatase activity is primarily localized in SCs and Leydig cells [53]. It has been known that estrogens have an essential role in regulating the HPT axis and thus indirectly regulate LH and testosterone equilibrium through a feedback loop [54]. Besides from gonadotrophins and steroid hormones, thyroid hormone has also been shown to play an important role in testicular physiology [55, 56]. Thyroid gland produces thyroxine (T4) and tri-iodothyronine (T3) [56]. It is known that T3 regulates the maturation and growth of testis, in rats and other mammalian species, by inhibiting immature SCs proliferation and by stimulating their functional differentiation [57, 58]. Likewise, thyroid hormone has been shown to play a critical role in the onset of Leydig cell differentiation and stimulation of steroidogenesis in postnatal rat testis [56]. Though the mechanisms implicated in the regulatory actions of thyroid hormone in testicular cells are still undefined, the presence of thyroid hormone receptors in human and rat testis throughout development and in adulthood implies that T3 may act via the classical genomic pathway in testis [59, 60].

Insulin-like growth factor I (IGF-1) is also biosynthesized in the testis by SCs [61], its receptors are identified in Leydig cells, peritubular cells, and spermatocytes [2]. It is known that IGF-1 stimulates the proliferation of Leydig cell precursors, spermatogenesis and spermatid maturation [61]. LH, FSH and testosterone may cooperate in spermatogenesis through stimulation of IGF-1 secretion by SCs [2, 61]. Growth hormone (GH) is not classically considered as a reproductive hormone, although it has important roles in reproductive function. It plays a role in steroidogenesis and spermatogenesis exerting an endocrine action either directly at gonadal sites or indirectly via IGF-1. GH influences Leydig cell steroidogenesis by regulating the secretion of IGF-1 and by increasing the expression of several genes that code for steroidogenic enzymes, including 3β-hydroxysteroid dehydrogenase, responsible for the conversion of pregnenalone to progesterone [2, 62-64]. Progesterone is another hormone very relevant for male reproduction. The maturation of progesterone to androstenedione is catalyzed by 17α-hydroxylase/ C17–20 lyase, while further conversion of androstenedione to testosterone depends on 17β-hydroxysteroid dehydrogenase activity, in Leydig cell smooth endoplasmic reticulum [2]. Therefore, it is essential to unravel the complex hormonal network

and signalling involved in the control of spermatogenesis to understand all the mechanisms relevant to male fertility, and unveil its flaws in cases of infertility.

References

1. Setchell, B.P., S. Maddocks, and D.E. Brooks, 1994, Anatomy, vasculature, innervation, and fluids of the male reproductive tract. The Physiology of Reproduction, 1: p. 1063-1175.
2. Niederberger, C., An Introduction to Male Reproductive Medicine. 2011, Cambridge University Press.
3. Weinbauer, G.F., et al., Physiology of testicular function, in Andrology, E. Nieschlag, H.M. Behre, and S. Nieschlag, Editors. 2010, Springer Berlin Heidelberg. p. 11-59.
4. Haider, S.G., 2004, Cell biology of Leydig cells in the testis. International Review of Cytology, 233: p. 181-241.
5. Ge, R. and M.P. Hardy, Regulation of Leydig cells during pubertal development, in The Leydig cell in health and disease, A.H. Payne and M.P. Hardy, Editors. 2007, Springer. p. 55-70.
6. Setchell, B.P., 1986, The movement of fluids and substances in the testis. Australian Journal of Biological Sciences, 39(2): p. 193-208.
7. Schell, C., et al., 2010, 15-Deoxy-Δ12-14-prostaglandin-J2 induces hypertrophy and loss of contractility in human testicular peritubular cells: implications for human male fertility. Endocrinology, 151(3): p. 1257-1268.
8. Romano, F., et al., 2005, The contractile phenotype of peritubular smooth muscle cells is locally controlled: possible implications in male fertility. Contraception, 72(4): p. 294-297.
9. Welsh, M., et al., 2009, Androgen action via testicular peritubular myoid cells is essential for male fertility. The FASEB Journal, 23(12): p. 4218-4230.
10. Schell, C., et al., 2008, Exploring human testicular peritubular cells: Identification of secretory products and regulation by tumor necrosis factor-α. Endocrinology, 149(4): p. 1678-1686.
11. Mayerhofer, A., 2013, Human testicular peritubular cells: more than meets the eye. Reproduction, 145(5): p. R107-R116.
12. Flenkenthaler, F., et al., 2014, Secretome analysis of testicular peritubular cells: a window into the human testicular microenvironment and the spermatogonial stem cell niche in man. Journal of Proteome Research, 13(3): p. 1259-1269.
13. Albrecht, M., et al., 2006, Isolation and cultivation of human testicular peritubular cells: a new model for the investigation of fibrotic processes in the human testis and male infertility. The Journal of Clinical Endocrinology & Metabolism, 91(5): p. 1956-1960.

14. Mruk, D.D. and C.Y. Cheng, 2004, Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocrine reviews, 25(5): p. 747-806.
15. Zhengwei, Y., et al., 1998, Stereological Evaluation of Human Spermatogenesis after Suppression by Testosterone Treatment: Heterogeneous Pattern of Spermatogenic Impairment 1. The Journal of Clinical Endocrinology & Metabolism, 83(4): p. 1284-1291.
16. Rato, L., et al., 2012, Metabolic regulation is important for spermatogenesis. Nature Reviews Urology, 9(6): p. 330-338.
17. Madara, J.L., 1998, Regulation of the movement of solutes across tight junctions. Annual Review of Physiology, 60(1): p. 143-159.
18. Alves, M.G., et al., 2013, Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cellular and Molecular Life Sciences, 70(5): p. 777-793.
19. Pelletier R. The tight junctions in the testis, epididymis, and vas deferens. In: Cereijido M, Anderson J (eds). Tight Junctions. New York: CRC Press, 2001, 599 –628.
20. Griswold, M.D., 1998, The central role of Sertoli cells in spermatogenesis. Seminars in Cell & Developmental Biology, 9(4): p. 411-416.
21. Fritz, I.B., et al., 1976, Regulation by FSH and dibutyryl cyclic AMP of the formation of androgen-binding protein in Sertoli cell-enriched cultures. Journal of Reproduction and Fertility, 46(1): p. 17-24.
22. Skinner, M.K. and M.D. Griswold, 1980, Sertoli cells synthesize and secrete transferrin-like protein. Journal of Biological Chemistry, 255(20): p. 9523-9525.
23. Marzowski, J., et al., 1985, Isolation and characterization of Sertoli cell plasma membranes and associated plasminogen activator activity. Biology of Reproduction, 32(5): p. 1237-1245.
24. O'Brien, D.A., C.A. Gabel, and E.M. Eddy, 1993, Mouse Sertoli cells secrete mannose 6-phosphate containing glycoproteins that are endocytosed by spermatogenic cells. Biology of Reproduction, 49(5): p. 1055-1065.
25. Elkington, J. and I. Fritz, 1980, Regulation of Sulfoprotein Synthesis by Rat Sertoli Cells in Culture. Endocrinology, 107(4): p. 970-976.
26. Robinson, R. and I.B. Fritz, 1979, Myoinositol biosynthesis by Sertoli cells, and levels of myoinositol biosynthetic enzymes in testis and epididymis. Canadian journal of biochemistry, 57(6): p. 962-967.
27. Oliveira, P.F., et al., 2012, Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta (BBA)-General Subjects, 1820(2): p. 84-89.

28. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. The Journal of Membrane Biology, 236(2): p. 215-224.
29. Rato, L., et al., 2011, Blood testis barrier: how does the seminiferous epithelium feed the developing germ cells. Endothelium and Epithelium: Composition, Functions and Pathology. Nova Biomedical: New York, NY: p. 137-155.
30. Sharpe, R.M., 1994, Regulation of spermatogenesis. The Physiology of Reproduction, 1: p. 1363-1434.
31. Cheng, C.Y. and D.D. Mruk, Spermatogenesis, mammals, in Brenner's Encyclopedia of Genetics (Second Edition), S. Maloy and K. Hughes, Editors. 2013, Elsevier. p. 525-528.
32. Cheng, C.Y., et al., 2010, Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Molecular and Cellular Endocrinology, 315(1): p. 49-56.
33. Hess, R.A. and L.R. de Franca, Spermatogenesis and cycle of the seminiferous epithelium, in Molecular mechanisms in spermatogenesis. 2008, Springer. p. 1-15.
34. Dym, M., 1994, Spermatogonial stem cells of the testis. Proceedings of the National Academy of Sciences of the United States of America, 91(24): p. 11287.
35. Hofmann, M.-C., 2008, Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Molecular and Cellular Endocrinology, 288(1): p. 95-103.
36. Sá, R., et al., 2013, Expression of stem cell markers: OCT4, KIT, ITGA6, and ITGB1 in the male germinal epithelium. Systems biology in reproductive medicine, 59(5): p. 233-243.
37. Sá, R., et al., 2008, Cytological and expression studies and quantitative analysis of the temporal and stage-specific effects of follicle-stimulating hormone and testosterone during cocultures of the normal human seminiferous epithelium. Biology of reproduction, 79(5): p. 962-975.
38. Rowley, M.J., J.D. Berlin, and C.G. Heller, 1971, The ultrastructure of four types of human spermatogonia. Zeitschrift für Zellforschung und mikroskopische Anatomie, 112(2): p. 139-157.
39. Grootegoed, J.A., M. Siep, and W.M. Baarends, 2000, Molecular and cellular mechanisms in spermatogenesis. Best Practice & Research Clinical Endocrinology & Metabolism, 14(3): p. 331-343.
40. Phillips, B.T., K. Gassei, and K.E. Orwig, 2010, Spermatogonial stem cell regulation and spermatogenesis. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1546): p. 1663-1678.
41.Knobil, E. and J.D. Neill, The physiology of reproduction.1994, Raven Press, p. 1- 3250.
42. Meistrich, M.L., et al., 1992, Highly acetylated H4 is associated with histone displacement in rat spermatids. Molecular Reproduction and Development, 31(3): p. 170-181.

43. Walker, W.H. and J. Cheng, 2005, FSH and testosterone signaling in Sertoli cells. Reproduction, 130(1): p. 15-28.
44. Habert, R., H. Lejeune, and J.M. Saez, 2001, Origin, differentiation and regulation of fetal and adult Leydig cells. Molecular and Cellular Endocrinology, 179(1): p. 47-74.
45. Rossi, P., et al., 1991, Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. Biochemical and Biophysical Research Communications, 176(2): p. 910-914.
46. Tilbrook, A.J. and I.J. Clarke, 2001, Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males. Biology of Reproduction, 64(3): p. 735-742.
47. Hill, J.W., J.K. Elmquist, and C.F. Elias, 2008, Hypothalamic pathways linking energy balance and reproduction. American Journal of Physiology-Endocrinology and Metabolism, 294(5): p. E827-E832.
48. Wade, G.N., J.E. Schneider, and H. Li, 1996, Control of fertility by metabolic cues. American Journal of Physiology-Endocrinology And Metabolism, 270(1): p. E1-E19.
49. Trumble, B.C., et al., 2010, Responsiveness of the reproductive axis to a single missed evening meal in young adult males. American Journal of Human Biology, 22(6): p. 775-781.
50. Saradha, B. and P.P. Mathur, 2006, Effect of environmental contaminants on male reproduction. Environmental toxicology and pharmacology, 21(1): p. 34-41.
51. Nindl, B.C., et al., 2001, LH secretion and testosterone concentrations are blunted after resistance exercise in men. Journal of Applied Physiology, 91(3): p. 1251-1258.
52. Carreau, S. and R.A. Hess, 2010, Oestrogens and spermatogenesis. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1546): p. 1517-1535.
53. Inkster, S., W. Yue, and A. Brodie, 1995, Human testicular aromatase: immunocytochemical and biochemical studies. The Journal of Clinical Endocrinology & Metabolism, 80(6): p. 1941-1947.
54. O'Donnell, L., et al., 2001, Estrogen and Spermatogenesis 1. Endocrine reviews, 22(3): p. 289-318.
55. Cooke, P.S., et al., 2004, Thyroid hormone, glucocorticoids, and prolactin at the nexus of physiology, reproduction, and toxicology. Toxicology and Applied Pharmacology, 194(3): p. 309-335.
56. Mendis-Handagama, S.M.L.C. and H.B.S. Ariyaratne, 2005, Leydig cells, thyroid hormones and steroidogenesis. Indian Journal of Experimental Biology, 43(11): p. 939-962.
57. Cooke, P.S., et al., 1991, Increased Sperm Production in Adult Rats After Transient Neonatal Hypothyroidism. Endocrinology, 129(1): p. 244-248.

58. Hess, R., et al., 1993, Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. Endocrinology, 132(6): p. 2607-2613.
59. Benbrook, D. and M. Pfahl, 1987, A novel thyroid hormone receptor encoded by a cDNA clone from a human testis library. Science, 238(4828): p. 788-791.
60. Jannini, E.A., et al., 1990, Ontogenesis of the nuclear 3, 5, 3'-triiodothyronine receptor in the rat testis. Endocrinology, 126(5): p. 2521-2526.
61. Itoh, N., et al., 1994, Restoration of Testicular Transferring Insulin-Likegrowth Factor-1 (IGF-1), and Spermatogenesis by Exogenously Administered Purified FSH and Testosterone in Medically Hypophysectomized Rats. Systems Biology in Reproductive Medicine, 33(3): p. 169-177.
62. Gomez, J.M., et al., 1999, Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (Oncorhynchus mykiss). General and Comparative Endocrinology, 113(3): p. 413-428.
63. Spiteri-Grech, J. and E. Nieschlag, 1992, The role of growth hormone and insulin-like growth factor I in the regulation of male reproductive function. Hormone Research in Paediatrics, 38(Suppl. 1): p. 22-27.
64. Hull, K.L. and S. Harvey, 2000, Growth hormone: a reproductive endocrine-paracrine regulator? Reviews of Reproduction, 5(3): p. 175-182.

pH and male fertility: making sense on pH homeodynamics throughout the male reproductive tract

Abstract

In the male reproductive tract, ionic equilibrium is essential to maintain normal spermatozoa production and, hence, the reproductive potential. Among the several ions, HCO_3^- and H^+ have a central role, mainly due to their role on pH homeostasis. In the male reproductive tract, the major players in pH regulation and homeodynamics are carbonic anhydrases (CAs), HCO_3^- membrane transporters (solute carrier 4 -SLC4 and solute carrier 26 - SLC26 family transporters), Na^+-H^+ exchangers (NHEs), and monocarboxylate transporters (MCTs). CAs and these membrane transporters are widely distributed throughout the male reproductive tract, thus playing essential roles in the ionic balance of tubular fluids. CAs are the enzymes responsible for the production of HCO_3^- which is then transported by membrane transporters to ensure the maturation, storage and capacitation of the spermatozoa. The transport of H^+ is carried out by NHEs and MCTs and is essential for the electrochemical balance and for the maintenance of the pH within the physiological limits along the male reproductive tract. Alterations in HCO_3^- production and transport of ions have been associated with some male reproductive dysfunctions. Herein, we present an up-to-date review on the distribution and role of the main intervenient on pH homeodynamics in the fluids throughout the male reproductive tract. In addition, we discuss their relevance for the establishment of the male reproductive potential.

Keywords: Spermatogenesis; Sperm; pH; Carbonic anhydrases; MCTs; NHEs

Introduction

A decline in reproductive health has been observed over the last few decades. Worldwide, approximately one in five couples are infertile, and male infertility contributes to about 50% of these cases [1]. Male reproductive potential requires a high level of cellular cooperation in an extensively balanced network. Sperm production, maturation, storage, and transport is a complex multi-step process that requires a tightly regulated environment [2-5]. Spermatozoa are produced in the enclosed environment of the seminiferous tubules with the support of the somatic Sertoli cells (SCs). After spermatogenesis occurs within the seminiferous tubules, spermatozoa are transported to the *rete testis* and from there across the efferent ducts to the

epididymis where are stored [6]. Throughout this process, spermatozoa are subjected to sequential changes in the luminal fluid composition, beginning in the seminiferous tubules and all the way to the seminal fluid. These changes in composition are not only the result of different protein secretory profiles, according to the anatomical region, but also of changes in membrane proteins that contribute to water resorption and ion transport [7, 8]. Electrolytes and water are essential components of the seminal fluid. The formation of the ionic composition of the luminal fluid includes the net movement of water, Cl^-, Na^+, and HCO_3^- reabsorption that contribute to fluid pH homeodynamics [9]. In addition, spermatozoa development, maturation and storage is also depend on a range of proteins (enzymes, membrane transporters,…) that participate in pH regulation [9-11] illustrating the relevance of pH homeostasis for those processes. Indeed, alterations in luminal fluids pH have severe effects on the entire reproductive process, from spermatogenesis to sperm capacitation and sperm capacity fertilization [7]. The pH of the fluids is strongly correlated with electrolytic composition, so it is essential to understand ionic production and transport in the male reproductive tract fluids [8]. In this review, we will address some of the players that contribute to the maintenance of intra- and extracellular pH within the appropriate physiological range in the male reproductive tract. We will particularly focus on HCO_3^- and in the main proteins responsible for its transport in the male reproductive tract, and enzymes that contribute to its production, such as carbonic anhydrase. We will also discuss how Na^+ and H^+ transporters contribute to the maintenance of pH as well as the role of monocarboxylate transporters (MCTs) that are so important for the transport of essential metabolites accompanied by H^+ and thus also contribute to the pH homeostasis of the fluids throughout the male reproductive tract.

Relevance of luminal pH homeostasis along the male reproductive tract

Homeostasis of the luminal fluid pH is pivotal for the entire reproductive process, including to spermatogenesis, sperm maturation and capacitation, fertilization of the oocyte and embryonic development [7]. The composition of the luminal fluid, and consequently the pH, varies along the male reproductive tract, in order to create an optimal environment for the acquisition of functional spermatozoa characteristics [8].

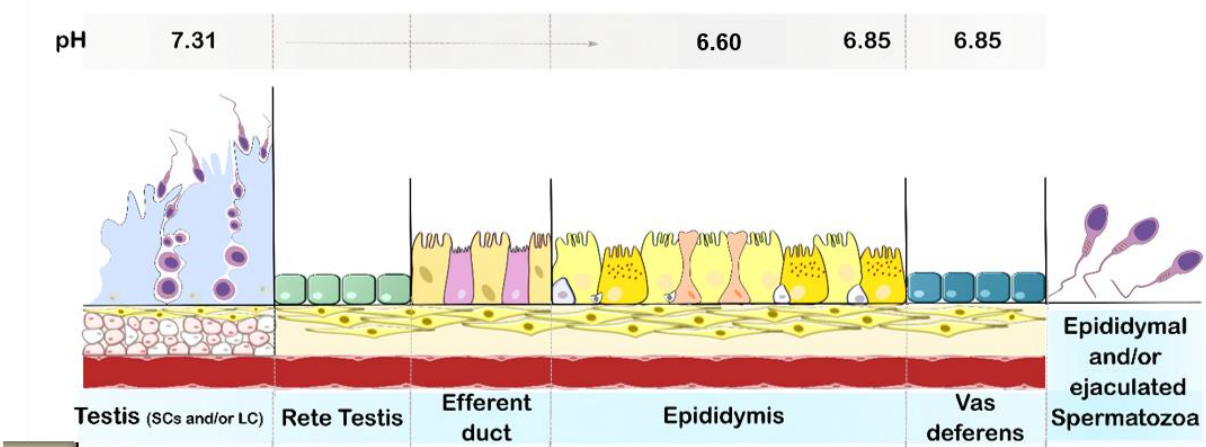
The luminal fluid that surrounds the spermatozoa begins to be produced inside the seminiferous tubules, where spermatogenesis occurs. The fluid leaving the testis is a suspension consisting of the spermatozoa released into the lumen of seminiferous tubule and fluids originated in the testis, particularly by SCs [12]. The seminiferous tubular fluid (STF) is characterized by a more acidic pH (≈7.31) compared to the plasma pH (≈7.5) [13, 14]. To maintain this pH is essential a net movement of water, Cl^-, Na^+ and HCO_3^- reabsorption and K^+ secretion [9]. STF and spermatozoa are driven through the *rete testis* and efferent ducts,

and the fluid becomes sodium chloride-rich instead of potassium as in the testis [15]. The majority of the testicular fluid is reabsorbed in the efferent ducts, reaching the initial segment of the epididymis as a fluid with less water content and a higher concentration of spermatozoa and ions [16]. However, little is known concerning luminal fluid control in *rete testis* and efferent ducts, largely due to the difficulties of micropuncture techniques and the reduced volume of fluid that can be analyzed.

From the time they leave the testis until they reach the end of the epididymis, spermatozoa are bathed by an ever-changing fluid, due to alterations in protein content like other chemical components [17]. These changes are essential for the maturation and quiescence state of the spermatozoa in the male reproductive tract. Indeed, the mammalian epididymis is a complex tubule that is structurally divided into three regions: caput, corpus and cauda. Notably, each region presents different properties with slight variations in the pH of the fluid, depending on the state of maturation of spermatozoa [18] (Figure 1.2). The fluid that fills the inside of the epididymis is controlled by the blood-epididymal barrier (BEB), which maintains a controlled luminal fluid, creating an appropriate environment for sperm maturation and survival [19]. The epididymal fluid is hyperosmotic when compared to the plasma and it is rich in inorganic ions and small organic molecules [20]. Alterations in the constitution of the epididymal fluid can lead to changes in pH and thus affect the maturation and development of spermatozoa [7]. In caput and corpus regions occurs the principal maturational events for spermatozoa to acquire motility and fertilizing ability [21]. Maturation implicates biochemical and morphological alterations in the sperm surface caused by epididymal secretions of proteins, glycoproteins, enzymes and ionic molecules that will constitute the tubular fluid and will have an essential role for the process of fertilization [22]. The distal cauda is a storage site for functionally mature spermatozoa in a quiescent state, but viable, state until ejaculation [23]. Since they can be stored for several days until ejaculation, the components of this epididymal microenvironment should be strictly controlled. Levine and Kelly [14] have reported that the slightly acidic pH found in epididymal fluid is essential for maintaining sperm viability and quiescent state during epididymal storage. This implies that there should be a control in the composition of epididymal luminal fluid, which includes a complex mixture of water, inorganic ions, small organic molecules and proteins [24].

In mammals, the fluid pH is significantly lower in the epididymis than in the testis, but is again elevated in the ejaculate. The seminal plasma that forms the ejaculated is a fluid resultant from the mixture of fluids derived from the testes, epididymis, prostate, and seminal vesicles secretions [7]. The pH in seminal plasma is maintained around 7.2 -8.4 in humans, essentially by HCO_3^- produced by accessory reproductive glands [13, 25]. During ejaculation and passage to the female reproductive tract, the alterations on pH and HCO_3^- concentration induces sperm

capacitation [26, 27] through activation of soluble adenylate cyclase (sAC) that is responsible for the passage from immature to mature spermatozoa [28]. So, HCO_3^- present in the seminal fluid is the principal factor for initial sperm motility at the time of ejaculation [7]. The luminal fluid in the vagina is generally acidic, where the seminal fluid is supposed to function as a buffer to control pH changes. However, the fluid in the uterus and oviduct is alkaline and contains a high concentration of HCO_3^-, that provides a second phase to sperm capacitation [7, 29]. Thus, from formation of spermatozoa until fertilization, pH homeostasis throughout male reproductive tract is essential.



	Testis (SCs and/or LC)	Rete Testis	Efferent duct	Epididymis	Vas deferens	Epididymal and/or ejaculated Spermatozoa
CAs	CA IV	CA IX	CA IX CAXII	CA II CA IV CA III CA XII CA XIV	N.D.	CA IV CA II
HCO_3^- Transporters	AE1 AE2 AE3 NBCe1 NBCn1 NDCBE NCBE SLC26A4 SLC26A7	N.D.	SLC26A4 SLC26A7	AE2 NBCe1 NBCe2 NBCn1 NDCBE SLC26A4 SLC26A7	AE2 NDCBE SLC26A4 SLC26A7	AE2 SLC26A3 SLC26A6 SLC26A8
NHEs	NHE2 NHE3 NHE8	N.D.	NHE1 NHE2 NHE3	NHE1 NHE2 NHE3	NHE1	NHE1 sNHE
MCTs	MCT1 MCT2 MCT4	N.D.	MCT1	MCT1	N.D.	MCT2

Figure 1.2 Principal enzymes and membrane transporters that regulate the pH in the male reproductive tract and spermatozoa. This figure summarizes the isoforms of carbonic anhydrase (CAs), HCO_3^- transporters, Na^+-H^+ exchangers (NHEs) and monocarboxylates transporters (MCTs) described along the male reproductive tract. The pH of luminal fluids varies along the male reproductive tract, in seminiferous tubular lumen is about 7.31 becoming more acidic in the epididymis (caput - 6.60, tail - 6.85) and vas defferens (6.85). Abbreviations: SCs – Sertoli cells; LC – Leydig cells; AE – Anion exchanger; NBCe - Na^+/HCO_3^- cotransporter electrogenic; NBCn - Na^+/HCO_3^- cotransporter electroneutral; NDCBE - Na^+-dependent Cl^-/HCO_3^- exchangers; SLC – Solute carrier; NCBE - Na^+-coupled Cl^- and HCO_3^- exchanger. N.D. – Not defined

Carbonic anhydrases: In brief

The concentration of HCO_3^- and CO_2, the components of a carbonic buffer, are directly related with the maintenance of a physiological pH in tissues and cells, which controls the existence of body fluids with an appropriate pH [30]. Carbonic anhydrases (CAs) are metalloenzymes that catalyse this physiological reaction, the reversible hydration of CO_2 to H_2CO_3, being also able to catalyse a variety of other secondary reactions (Figure 1.3). CAs were initially purified from red blood cells [31] and subsequently identified in almost all organs, where they are implicated in some biological functions. They have an important participation in the regulation of ion, water and acid-base equilibrium in the body [32]. CAs are implicated in a net acid/base transepithelial transport in some epithelia and ducts, including epididymal ducts [33, 34]. Some isoforms are regulators of cell proliferation and tissue maturation, including with great relevance in the uncontrolled cell proliferation that takes place in cancer [35].

To date, 14 different CA isoforms have been described in higher vertebrates, including humans [36]. They are divided by kinetic and chemical properties, with focus in their relative abilities to catalyse the reactions between CO_2, HCO_3^- and water. CA II, CA IV, CA VII and CA IX are the isoforms with major catalytic activity when concerning CO_2 hydration [37]. Several CAs isoforms are expressed in multiple ion-transporting epithelial tissues, with different subcellular localizations. CA I, CA II, CA III, CA VII are cytosolic isoforms, while there are some isoforms just cell membrane-bound (CA IV, CA IX, CA XII and CA XIV). CA V is the only isoform present in mitochondria [37] and CA VI is secreted into saliva and milk [38]. Concerning CA VIII, CA X, CA XI, and CA XIII, there are still no studies confirming its subcellular location (Figure 1.3). Overall, CAs have several physiological functions and are involved in many cellular functions.

Carbonic anhydrase and HCO_3^- in male reproductive tract

The pH of luminal fluids is a central feature for the determination of male reproductive potential [39]. HCO_3^- concentration is a major player that helps to maintain this parameter within suitable values, in order to promote the adequate spermatozoa development. Homeostasis of acid-base balance of the luminal pH will be maintained by the action of the carbonic buffer and consequently by the intervention of CAs [30].

The role of CAs in the male reproductive tract is not yet fully understood, although the multiple CA isoforms have been identified in the various cells of the reproductive tissues. Indeed, histochemical observations showed that CAs are specifically present throughout the entire male reproductive tract [40] (Figure 1.2).

CA IV is present in rat testis, specifically in capillary endothelium and in the membrane of peritubular myoid cells surrounding the seminiferous epithelium [41] (Figure 1.2). This

membrane-bound isoform is also present in mouse spermatozoa collected from corpus and cauda, but was not detected in spermatozoa collected from testis and caput, [41], thus suggesting that the protein is synthesized by the epididymal epithelium and transferred to the spermatozoa. Since CA IV is linked to the membrane by a glycosylphosphatidylinositol, the protein is transferred to the membrane when spermatozoa pass through the duct [42]. High activity of CA II was detected also in human and rat spermatozoa (Figure 1.2). In rats, CA II is located in the acrosomal cap region and in humans it is also detected in the head of the spermatozoa, between the acrosomal region and the neck [43]. It is estimated that this high enzymatic activity maintains the concentration of intracellular HCO_3^-, and thus sperm motility.

Spermatozoa acquire motility and fertilising ability during the passage by the epididymal duct. The spermatozoa are kept until ejaculation in a quiescent state in the epididymis tail, where the control of ionic compounds is essential for maintenance of a suitable pH. It is known that acidic pH of epididymal fluid inhibits sperm motility [44], while an increase in pH and/or HCO_3^- concentration stimulates sperm motility [45, 46]. Altering the HCO_3^- concentration in the fluid can control spermatic motility by two pathways: by intracellular acidification; but also by regulation of HCO_3^--sensitive adenylate cyclase in the sperm plasma membrane [46].The mechanism that leads to the acidification of the epididymal fluid is not completely clear, but it is known that CAs plays an important role in this process, since intravenous infusions of acetazolamide, a general inhibitor of CAs, significantly decrease acidification in the epididymis of rats [47]. The inhibition of CAs by injection of acetazolamide also led to decreased secretion of testicular fluid and consequently reduction in the production of seminal plasma [48].

In fact, several CA isoforms have been described in rat epididymis, namely CA II, CA III, CA IV, CA XII, and CA XIV [49] (Figure 1.2). The localization of these isoforms suggests a role in pH control of epididymal fluid, being present both in the cytosol and apical plasma membrane of the epididymal cells [50]. CA activity was detected in epithelial cells of the initial segment, caput, corpus and cauda of the epididymis [41]. In the initial segment and caput, CAs are found apical cell membranes, where the amount of HCO_3^- is reduced and the epididymal fluid is acidic. In the cauda, CA activity was observed in clear cells. This distal region of the epididymis is characterized by a more alkaline pH than in the initial segment, and it was suggested that clear cells participate in the process of alkalinisation due to the capacity of CAs to produce HCO_3^- [13, 33]. The presence of CAs then gradually decreases between the epididymis and the vas deferens [41].

Efferent ducts are responsible for absorption up to 96% of the testicular fluid and fluid absorption is directly correlated to transepithelial ion transport [51]. CAs are expressed in several regions of this duct. For instance, CA IX and CA XII are identified in the basolateral plasma membranes of the epithelial cells of the efferent ducts [52] (Figure 1.2). It is suggested

that the role of these two isoenzymes is related to transepithelial ion transport and water absorption in the duct. The absorption of Cl^- and Na^+ ions from the lumen of the efferent ducts is coupled with HCO_3^- transport by the solute carrier transporters (SLC), which cotransport Cl^-/HCO_3^- and Na^+/ HCO_3^- [39, 53]. Thus, CAs present in efferent ducts may control the accumulation of HCO_3^- by the intervention of the SLC transporters [52]. In human *rete testis* it was also detected the expression of CA IX, where its function is still unclear [54]. Altogether, CAs represent a potential regulator for the HCO_3^- homeostasis in the composition of the male genital tract fluids.

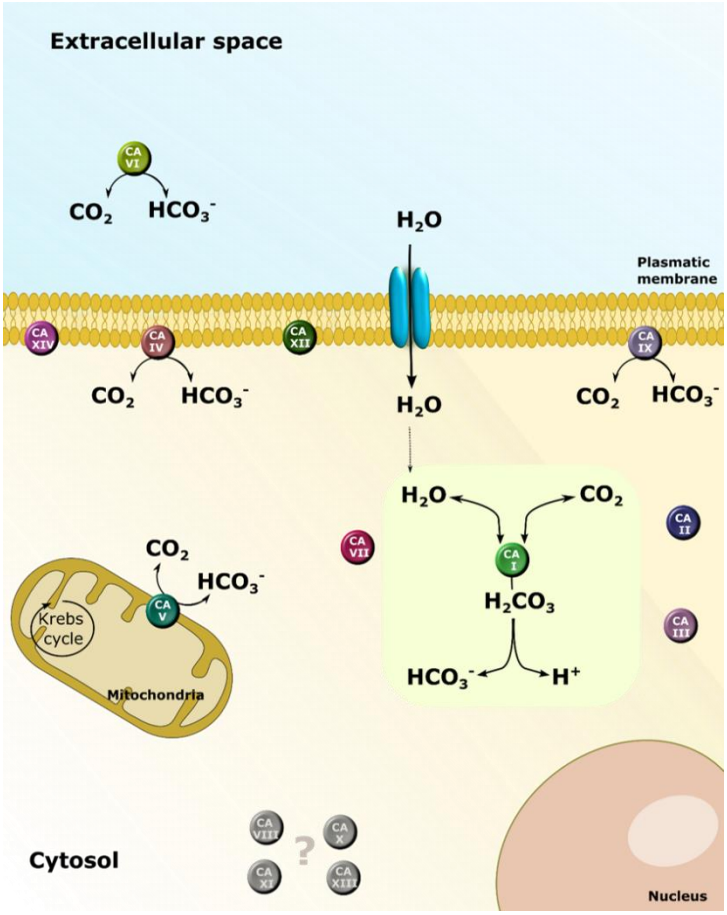


Figure 1.3 Schematic representation of the distribution of carbonic anhydrase (CA) isoforms in cells. CAs catalyse the reversible hydration of CO_2 to H_2CO_3 that then can dissociate into HCO_3^- and H^+. CA I, CA II, CA III, CA VII are cytosolic isoforms, and CA IV, CA IX, CA XII and CA XIV are cell membrane-bound isoforms. CA V is the isoform present in mitochondria, and CA VI is a secretable isoform. The subcellular location of CA VIII, CA X, CA XI, and CA XIII remains unclear.

HCO_3^- transporters in brief

In physiological conditions, the transport of HCO_3^- through the plasma membrane of cells is essential to maintain the ionic balance in the intra- and extracellular media of higher organisms [55]. Alterations in the concentration of HCO_3^- and CO_2 are reflected as pH variations, which plays a crucial role in organisms. Numerous chemical reactions and cellular enzymes are sensitive to pH, therefore cells actively transport HCO_3^- and CO_2 across their cellular membranes to maintain ionic equilibrium and consequently the pH [56, 57]. Regarding their functionality, HCO_3^- transporters essentially depend on the ionic gradient generated through the cellular membrane. They are classified as acid-extruders if they promote an increase of intracellular pH (pHi), for instance when acidosis occurs, or as acid loaders if they do the opposite transport, decreasing pHi when alkalosis occurs.

HCO_3^- membrane transporters are divided into two protein families, Solute Carrier 4 (SLC4) and Solute Carrier 26 (SLC26) [58, 59]. In addition, cystic fibrosis transmembrane conductance regulator (CFTR) is essentially a Cl^- channel, but has also been reported to be responsible for HCO_3^- conductance [60, 61]. All these transporters have a crucial role in the pH homeostasis throughout the male reproductive tract. We will briefly discuss their relevance in those processes.

SLC4 family: in brief

SLC4 family is the major group of membrane HCO_3^- transporters, comprising ten protein members. Eight family members have well-established functions, sharing the ability to transport basic particles, such as CO_2 and HCO_3^- and differing in the ability of the cotransport of Cl^- and/or Na^+ [61]. The members of this family of transport proteins have diverse functions, serving both as exchangers or cotransporters. The SLC4 protein family is usually separated into two functional groups due to its transport capacity, but all have in common the ability to transport HCO_3^-: Na^+- independent HCO_3^- transporters and Na^+-dependent HCO_3^- transporters [61] (Figure 1.4a).

Na^+- independent HCO_3^- transporters are responsible for mediating electroneutral exchange of Cl^- for HCO_3^- and/or CO_3^{2-}. This subfamily is constituted by three members of anion exchangers: solute carrier family 4 member 1 (SLC4A1) or anion exchanger 1 (AE1), SLC4A2 (or AE2) and SLC4A3 (or AE3). AE1 exchanges equal amounts of HCO_3^- for Cl^-, wherein the transporter can be either in an inward-facing or an outward-facing status [62]. This transport is regulated by a conformational change in the protein that transports a single ion across the cell membrane at a time [63]. AE2 is the anion exchanger more widely expressed in mammals, and it is usually known as a housekeeping of Cl^-/HCO_3^- transport. AE2 is an important pHi regulator, which is activated by exposure to alkaline environment and inhibited by exposure to

acidic environment [64, 65]. Like AE2, the transport performed by AE3 is also stimulated by an alkaline medium, however, this carrier has less affinity for Cl^-/HCO_3^- transport than the others Na^+- independent HCO_3^- transporters [62, 66] (Figure 1.4a).

Na^+-dependent HCO_3^- transporters encompasses the Na^+/HCO_3^- electrogenic (SLC4A4 or NBCe1 and SLC4A5 or NBCe2) and electroneutral cotransporters (SLC4A7 or NBCn1), the Na^+-dependent Cl^-/HCO_3^- exchangers (SLC4A8 or NDCBE) and the Na^+-coupled Cl^- and HCO_3^- exchanger (SLC4A10 or NCBE) [61, 67]. The role and mechanism of transport of SLC4A9 remains unclear, as there is considerable controversy in the literature regarding this transporter. The Na^+-dependent HCO_3^- transporters function predominantly as acid-extruders, that is, they function to increase pHi when acidosis occurs. These plasma membrane proteins transport Na^+ and HCO_3^- in the same direction, varying only in their stoichiometry [67]. The stoichiometry is $1Na^+:1HCO_3^-$ for the electroneutral transporter (NBCn1) and $1Na^+:2HCO_3^-$ or $1Na^+:3HCO_3^-$ for NCBe1 and NBCe2, respectively (Figure 1.4a).

The transport made by the electroneutral transporters is only due to the chemical gradient formed between the intra- and extracellular medium without an influence of the membrane potential. Under physiological conditions, the extracellular concentration of Na^+ is higher than the intracellular concentration, directing the entry of Na^+ and HCO_3^-, which functions as an acid extruder [68]. The electrogenic transporters (NBCe1 and NBCe2) are influenced by the electrical potential inside the cell, which is mostly negative. For example, in the pancreatic duct, NBCe1 directs the movement of Na^+ and HCO_3^- with 1:2 stoichiometry, favouring HCO_3^- uptake across the basolateral membrane for secretion into the lumen [69]. However, in the renal proximal tubule, NBCe1 mediates the efflux of HCO_3^- through the membrane into the blood. In this case, operating with stoichiometry 1:3, NBCe1 is essential for the exit of HCO_3^-, which makes the reversal potential for the transporter more positive than the basolateral membrane potential of the cells [70, 71] (Figure 1.4a).

NDCBE is able to transport extracellular Na^+ and HCO_3^- in exchange for intracellular Cl^- and H^+, being considered one of the major pHi regulator by its essential role in cellular alkalinisation [72, 73]. Human NDCBE makes an electroneutral transport by exchanging $1Cl^-$ for $1Na^+$ and $2HCO_3^-$ [74] (Figure 1.4a).

Na^+-coupled Cl^- and HCO_3^- exchanger (SLC4A10 – NCBE) is considered as an NCBn2 by most authors, because it is an electroneutral Na^+/HCO_3^- cotransporter with Cl^- self-exchange activity, since the efflux of Cl^- is not coupled to the influx of Na^+ and HCO_3^-, but a self-exchange of Cl^- stimulated by HCO_3^- and uncoupled to Na^+ [75] (Figure 1.4a).

SLC26 family: at brief

The SLC26 protein family consists of ten members with highly versatile anion exchangers. The SLC26 transporters are electrogenic, with diverse stoichiometry and anions specificity, which are able to transport Cl^-, HCO_3^-, hydroxyl ion (OH^-), sulfate, oxalate, formate, and iodide [59, 76-78] (Figure 1.4b).

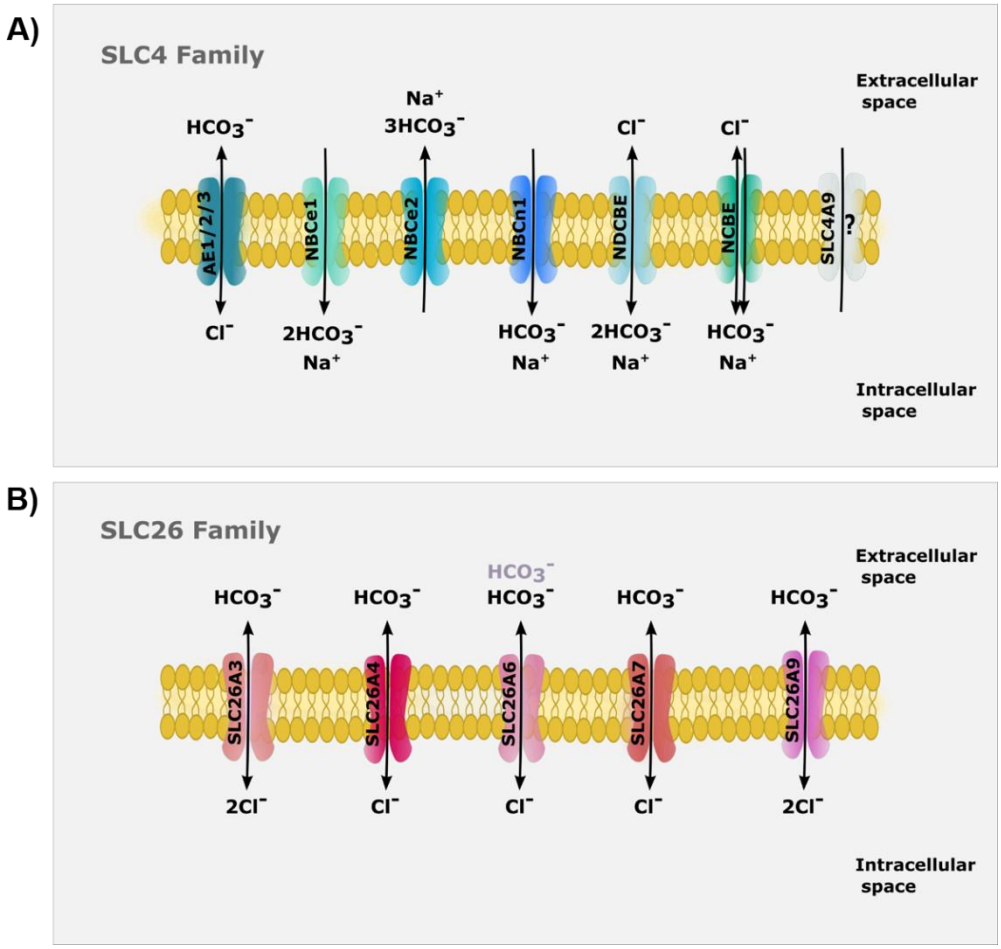


Figure 1.4 Principal families of bicarbonate transporters. **A)** Members of solute carrier 4 (SLC4) family. The anion exchangers 1, 2 and 3 (AE1/2/3) are Na^+ -independent Cl^-/HCO_3^- exchangers, mediating electroneutral exchange of HCO_3^- for Cl^- (1:1). The electrogenic Na^+/HCO_3^- cotransporters 1 and 2 (NBCe1 and NBCe2) operate with an apparent Na^+: HCO_3^- stoichiometry of 1:2 and 1:3, respectively. The electroneutral Na^+/HCO_3^- cotransporter 1 (NBCn1) is an electroneutral Na^+-dependent transporter, which transports Na^+ and HCO_3^- in the same direction with a stoichiometry of 1:1. The Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE) member mediates the electroneutral exchange of Cl^- for Na^+ and HCO_3^-. The Na^+-coupled Cl^- and HCO_3^- exchanger (NCBE) is an electroneutral Na^+/HCO_3^- cotransporter with Cl^- self-exchange activity and the mechanism of transport of SLC4A9 remains unclear. **B)** Members of solute carrier 26 (SLC26) family. SLC26A3 and SLC26A9 are an electrogenic Na^+-independent Cl^-- HCO_3^- exchangers, with an apparent stoichiometry of 2:1. SLC26A4 and SLC26A7 may be considered as electroneutral Cl^--HCO_3^- exchangers. SLC26A6 is an Cl^-- HCO_3^- exchanger that can operate with a stoichiometry of 1:2 or 1:1.

The exchangers differ in the anions they can transport. For instance, while the SLC26A6 is capable of transporting all the mentioned substrates, the SLC26A4 transports only monovalent anions (such as Cl^-, HCO_3^-, iodide, and formate) [77, 78].

Among the SLC26 family members, only five members are permeable to HCO_3^- in higher organisms: SLC26A3, SLC26A4, SLC26A6, SLC26A7, and SLC26A9 [77]. SLC26A3 mediates a coupled $2Cl^-$:$1HCO_3^-$ exchanger, being expressed in the apical membranes of epithelial cells and participating in HCO_3^- secretion and Cl^- reabsorption [79]. The original name of this member was DRA (down-regulated in adenomas), because its expression was shown to be down-regulated in adenomas [80]. In addition to being permeable to other substrates, SLC26A6, or PAT-1 (putative anion transporter-1), transports Cl^- and HCO_3^- with 1:2 or 1:1 stoichiometries [79, 81]. Moreover, SLC26A6 regulates CFTR activity in the pancreatic duct, thereby regulating pancreatic HCO_3^- secretion [82]. SLC26A7 shows a very restricted distribution and transports Cl^- and HCO_3^-, although the permeability to HCO_3^- is described as minimal. Thus, SLC26A7 seems to be a pHi-regulated Cl^- channel [83]. SLC26A7 is also able to transport sulfate and oxalate, which are essential in nephrons [84]. Finally, SLC26A9 is a Cl^-/HCO_3^- exchanger, in addition to transporting other solutes. Like SLC26A7, this transporter functions as a Cl^--channel with minimal HCO_3^- conductance [85]. Moreover, SLC26A9 also stimulates CFTR expression and function in lung [86] (Figure 1.4b).

Role for HCO_3^- transporters in determining male reproductive potential

HCO_3^- is essential to maintain pH in appropriated values along the male reproductive tract. As discussed, the male reproductive tract presents different fluids with distinct environments from the STF to the semen. Also, HCO_3^-concentration, and consequently pH, of the luminal fluids in male reproductive tract has intense effects throughout all processes associated with sperm and maturation, including spermatogenesis, sperm capacitation, fertilization in the female reproductive tract and in early-stage embryo development [39, 61].

The spermatozoa are generated in the testes and undergo alterations until their storage in the tail of the epididymis. During this course, spermatozoa are subjected to fluids with different pHs that are essential for their maturation and fertilization capacity. Since HCO_3^- is one of the major physiological buffers, the presence and the function of HCO_3^- membrane transporters along the male reproductive tract is an essential trait [39]. Indeed, micropuncture studies using rats established a direct correlation between pH and the concentration of HCO_3^- in fluids produced in the male reproductive tract. The lumen of the seminiferous tubules of rat testes presents a pH around 7.31 and 19.6 mequiv/L of HCO_3^- (Figure 1.2). In the caput of the epididymis, the pH decreases as well as the concentration of HCO_3^- (pH 6.6 and 2.7 mequiv/L

for HCO_3^-), whereas in the tail and vas deferens it slightly rises (pH 6.85 and 6.7 mequiv/L for HCO_3^-) [13] (Figure 1.2).

Several HCO_3^- membrane transporters have been detected in the male reproductive tract, with more relevance to members of the SLC4 family. AE2 is the anion exchanger with more predominance in the male reproductive tract. It is expressed in the testicular tissue [61], particularly in SCs [87], cells of the germ line [88] and mature spermatozoa [88]. This "housekeeping" protein is also detected along the epididymal duct [53, 61], vas deferens [89], prostate [53] and seminal vesicles [53] (Figure 1.2).

Concerning the anion exchangers, AE2 appears to be essential for the normal occurrence of spermatogenesis. Indeed, studies on AE2 knockout mice showed that animals are infertile due to a failure in spermiogenesis. These knockout mice presented just a few late spermatids and total absence of spermatozoa in seminiferous tubule. Additionally, the epididymis is also affected by the AE2 absence, since the mice showed squamous metaplasia of the epididymal epithelium [90]. Moreover, *in vitro* studies also reported that HCO_3^- transporters can be modulated by sex steroid hormones. For instance, rat SCs treated with elevated doses of estrogens presented an increased expression of AE2 and NBCn1. This increased expression was reflected in changes in the transcellular ion transport in SCs and thus, it may affect male fertility [87]. Contrary to AE2, which is abundantly expressed in the male reproductive tract, AE1 and AE3 are not expressed in most of the tissues of the male reproductive tract. AE1 has only been detected in Leydig cells [91] and AE3 in developing germ cells and seminal vesicle glandular cells [91] (Figure 1.2).

Na^+- dependent HCO_3^- transporters also have an essential role along the male reproductive tract to control pH in ducts and into the cells. NBCe1 expression has also been reported in epididymis [92], vas deferens [93], prostate [94], seminal vesicles [91], and testis [61]. NCBe1 is also expressed in rat and human SCs [87, 95] (Figure 1.2). The presence of NBCn1 has been detected throughout the whole male reproductive tract, being expressed in the testes (SCs) [61, 87], epididymis [61], prostate and seminal vesicles [91]. In addition, NBCe2 has been reported in testis, epididymis [96], prostate and seminal vesicles [93]. Like in other tissues, it was suggested that the function of these proteins is associated with HCO_3^- secretion, particularly in the epididymis [10] (Figure 1.2).

The NDCBE is strongly expressed in testis [61], particularly in SCs [87], in epididymis [61], vas deferens, prostate and seminal vesicles [91] (Figure 1.2). As previously discussed, this protein seems to have an important role on the control of the intracellular pH of SCs and on the regulation of ion transport in the epididymal duct. Contrastingly, NCBE shows little expression along the male reproductive tract, with only weak expression in the testis and Leydig cells are described [91, 97] (Figure 1.2).

Importantly, *in vitro* studies have demonstrated that the expression of NBCe1, NBCn1 and NDCBE is modulated by sex steroid hormones (specifically estrogens) in human SCs. SCs treated with elevated concentrations of estrogens presented an increased expression of these transporters with consequent increase of pHi and decrease in transcellular transport. NBCe1 and NBCn1 are located in the basal portion, while NDCBE is expressed in the apical portion of human SCs plasma membrane. So, NDCBE must play an essential role in cellular alkalinisation, transporting Na^+ and HCO_3^- from the adluminal compartment in exchange for intracellular Cl^-. On the other hand, NBCe1 and NBCn1 transport Na^+ and HCO_3^- from the basal compartment to the intracellular medium, also contributing to alkalinisation [95]. Modulation of these proteins by estrogen may thus compromise spermatogenesis, and consequently the reproductive potential of males. Through *in vivo* studies, Joseph and collaborators [98] have also shown that NBCe1 is influenced by estrogens. Estrogen receptor α (ERα) null mice are infertile with protein expression of NBCe1, CA XIV and Na^+-H^+ exchanger (NHE3) decreased in epididymal duct in relation to wild-type mice. These changes caused an increase in the pH of the epididymal lumen and consequently there is a decrease in sperm motility [98].

In what concerns the members of the SLC26 family, the current knowledge of their expression and function throughout the male reproductive tract is scarce. It is known that SLC26A4 and SLC26A7 are present in testes, epididymis, vas deferens and efferent ducts [99] (Figure 1). It has further been described a major interaction between some members of SLC26 family and CFTR, especially in spermatozoa, which has been linked to the control of specific sperm parameters. For instance, SLC26A8 is a sperm-specific anion exchanger that forms a SLC26A8-CFTR complex. SLC26A8 protein strongly stimulates the activity of CFTR in spermatozoa [100]. Moreover, the disruption of *SLC26A8* gene in male mice causes sterility due to severe structural defects in spermatozoa, a total lack sperm motility (asthenozoospermia) and impairment of capacitation [101]. Since CFTR is essential to sperm motility and capacitation [102, 103], SLC26A8-CFTR complex must be a key factor in the regulation of anion transport required for correct sperm motility and capacitation [100]. SLC26A3 and SC26A6 are also expressed in spermatozoa, having an essential role in the capacitation process. During sperm capacitation, the membrane potential hyperpolarizes due to an increase in the concentration of intracellular Cl^- [104]. The increase in Cl^- inlet can thus be mediated by these members of the SLC26 family [105]. SLC26A3 and SLC26A6 also interact with CFTR. Chen and collaborators [106] described that SLC26A3 works in parallel with CFTR in pig sperm. HCO_3^- and Cl^- are indeed necessary for sperm capacitation, leading to the involvement of SLC26A3 in transporting HCO_3^-, and CFTR providing the recycling pathway for Cl^- [106]. In addition to SLC26A3, some authors suggested that SLC26A3,

SLC26A6 and CFTR participate together in pHi increase in spermatozoa, an essential event during capacitation [104]. In fact, mutations in the *SLC26A3* gene give rise to congenital chloride diarrhoea (CLD), an autosomal recessive disease with a defect in the intestinal Cl^-/HCO_3^- exchange [107]. Men with CLD also showed alterations in Cl^-/HCO_3^- transport in the reproductive tract, presenting a high concentration of Cl^- and low pH in semen. Patients with this mutation are subfertile, with oligoasthenozoospermia and form spermatoceles [108].

Hence, the involvement of HCO_3^- in several aspects of the physiology of the male reproductive tract evidences its significance in the maintenance of the reproductive potential. Studies on the distribution and on the roles of various HCO_3^- membrane transporters further highlight the importance of this ion and of the mechanism responsible for its homeodynamis for the regular production of spermatozoa.

Na^+/H^+ exchangers at brief

Beyond HCO_3^-, H^+ movement between body compartments are also vital to keep the pH homeostasis. The main protein family that actively transports H^+ across cellular membranes is the solute carrier 9 (SLC9) family, more commonly named Na^+/ H^+ exchanger (NHEs) family. The NHEs are involved in the electroneutral exchange of extracellular Na^+ with intracellular H^+, according to the gradient created across membrane between intracellular and extracellular space [11]. The activity of NHEs is characterized by Michaelis-Menten kinetics, since the transport is strongly dependent of Na^+ external concentration, being that each NHE isoform differs in the affinity for extracellular Na^+ [109, 110].

The NHEs family is constituted by 14 members (NHE1 – NHE11), divided in 3 subgroups: plasma membrane isoforms (NHE1 – NHE5), endomembrane isoforms (NHE6 – NHE9), SLC9B (NHA1 and NHA2) and SLC9C (sNHE and NHE11) [11, 111]. The isoforms of NHEs can be distinguished by their kinetic properties, drug sensitivity, modulation by protein kinases and their tissue and cellular localization [112]. We will discuss those isoforms in the next subsections (Figure 1.5a).

Plasma membrane isoforms

NHE1 is frequently named as the “housekeeping” of NHE isoforms. This isoform is present in the plasma membrane of most mammalian cells. In polarized epithelial cells, NHE1 is primarily located in the basolateral membrane [113, 114]. The human NHE1 contains a hydrophobic N-terminal and a hydrophilic domain in C-terminus facing the intracellular medium that regulates its activity. The action of NHE1 is essential in mechanisms of cellular alkalinisation, since this protein is able to extrude H^+ derived from metabolism acidification and constitutes the major pathway for Na^+ influx into the cells [111]. Due to its wide expression, NHE1 is probably the

major isoform involved in intracellular pH regulation [115]. In fact, its absence or malfunction can lead to serious pathological conditions. NHE1 KO mice exhibited a decrease rate of postnatal growth and high mortality in newborn animals. In addition, these animals also present ataxia and epileptic seizures [116]. In samples of NHE1 KO brain, a decreased steady-state of intracellular pH (despite the presence of HCO_3^-) and an increased expression of other Na^+ channels were observed [117-119]. On the other hand, overexpression of NHE1 is associated with cardiac hypertrophy and heart failure [120]. Increased NHE1 activity is related with intracellular Na^+ accumulation, that activates Na^+/Ca^{2+} exchangers and induces an increase in intracellular Ca^{2+}, which can lead to cell death [121, 122]. Moreover, several studies also evaluated the role of NHE1 in cancer. It is known that NHE1 promotes tumorigenesis by numerous mechanisms, such as cell migration, invasion, and metastasis, among others [111].
NHE2 is a plasma membrane protein transporter that is expressed in several organs. When located in epithelial cells, it is predominantly located in the apical membrane [123, 124]. Like NHE1, NHE2 absence or malfunction is linked to pathological alterations. As a matter of fact, NHE2 KO mice have reduced viability of gastric cells and reduced net acid secretion [125]. Furthermore, the absence of NHE2 in mice results in histological abnormalities in the pituitary, suggesting that NHE2 can contribute to the transport of fluids in this region [126]. However, there is no known human disease related with alterations in this protein.
NHE3 is also a plasma membrane isoform, although it is also present in internal membranes, as this isoform recycles between the plasma membrane and the endosomal compartment [127, 128]. The role of NHE3 has been evidenced in the gastrointestinal and renal physiology. NHE3 KO mice present absorptive defects in both renal tubular epithelia and intestinal epithelia [129]. These animals presented decreased Na^+ and water reabsorption from all intestinal segments. NHE3 also mediates the reabsorption of great part of Na^+ and water in the proximal tubule. The H^+ is extruded in exchange for the influx of Na^+, the opposite of the cell's gradient. NHE3 is also able to recover HCO_3^- from pro-urine [130]. So, null NHE3 mice present a metabolic acidosis and alkaline urine [131, 132]. Moreover, a functional activity of NHE3 in endosomes is required for normal uptake of albumin [133]. It is known that vesicular transport along the endocytic pathway is dependent on a suitable luminal acidification [134]. KO NHE3 mice showed an alteration in albumin delivery to endosomes and lysosomes in renal cells, that resulted in tubular proteinuria [133, 135].
NHE4 is a plasma membrane transporter located in the basolateral side in epithelial cells, essentially in renal tubules, in the stomach, and in the small intestine and colon [111]. Studies with NHE4 KO mice demonstrated hypochlorhydria in association with histological alterations of the stomach [136]. The functional characterization of NHE4 KO mice is related with an imbalance within acid–base homeostasis, since these animals presented a metabolic acidosis

[137]. Like NHE3, NHE5 also is expressed in both the plasma membranes and endomembrane compartments [128] (Figure 1.5a). NHE5 is predominantly expressed in the brain, but its expression was also detected in the spleen, skeletal muscle, and testis [138, 139]. Unfortunately, the functions of this isoform are still unclear and thus, further studies will be needed to unveil its physiological relevance.

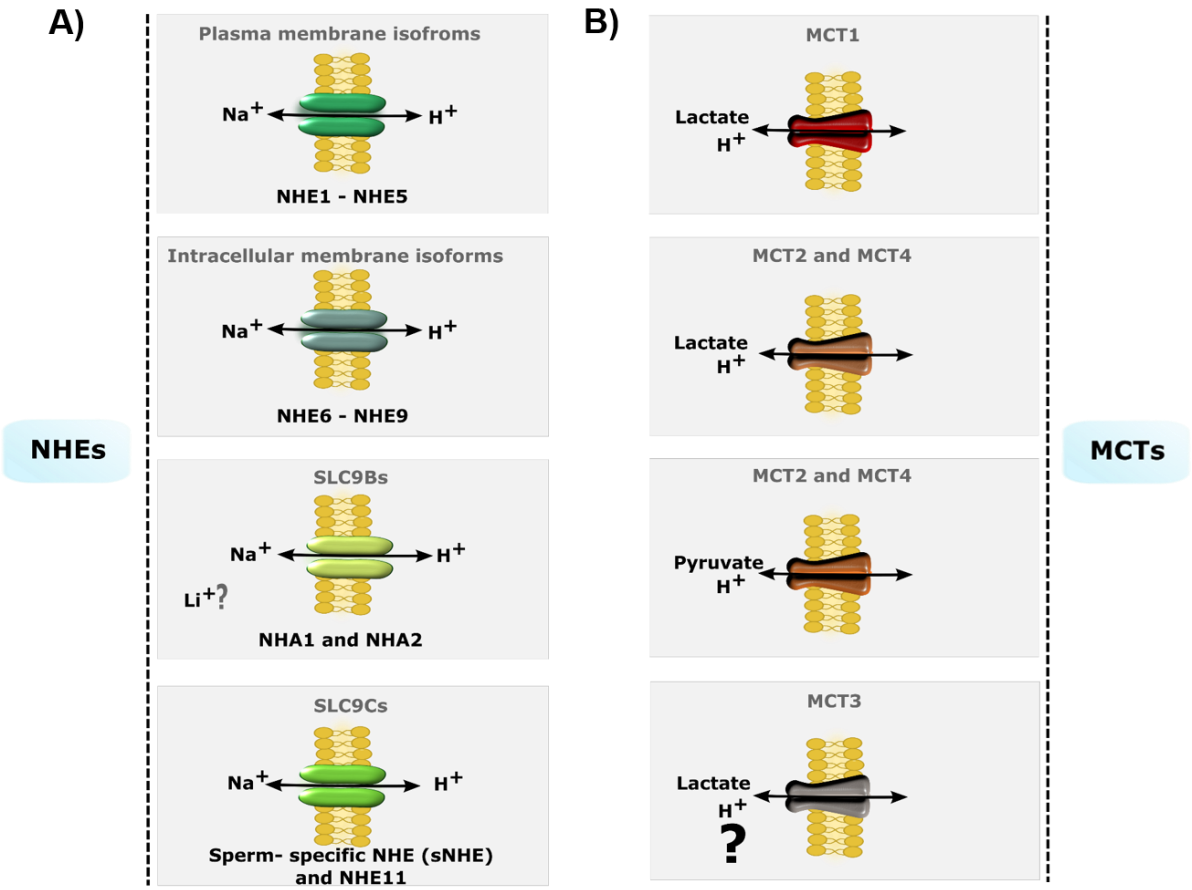


Figure 1.5 Schematic representation of the Na^+/ H^+ exchangers (NHEs or SLC9 family members) and monocarboxylates transporters (MCTs or SLC16 family members). **A)** NHE1 to NHE5 are plasma membrane isoforms and NHE6 to NHE9 are endomembrane isoforms. Solute carrier 9B (SLC9B) is constituted by two members – NHA1 and NHA2 and SLC9C is constituted by sNHE and NHE11, sNHE is a sperm-specific isoform and there are no data about NHE11 isoform. All these isoforms transport Na^+ in exchange for an H^+ (1:1). **B)** Four members of MCTs family are proton-coupled (MCT1-MCT4). MCT1 catalyses the net transport of one lactate with one H^+. MCT2 and MCT4 are able to transport lactate with H^+ or pyruvate with H^+. MCT3 it is suggested that transport lactate and H^+, but little is known about this transporter.

Intracellular NHEs

Endocytosis is the process by which plasmalemmal and extracellular compounds are internalized into the cell via endocytic vesicles and early endosomes. These compounds are then transported from the endosomes to other organelles of the cells. However, some material returns to the plasma membrane via recycling pathways [140]. Organellar pH is acidic and tightly controlled. The pH regulation of these organelles is essential for enzymatic activity regulation, membrane fusion, intracellular transport, post-translational alterations, and other changes [141]. For instance, pH gradually decreases from endosomes to lysosomes.

NHE6, NHE7, NHE8 and NHE9 are monovalent cation exchangers of the NHE family present on endosomes (Figure 1.5a). NHE6 is present in most cells, being localized mainly in intracellular compartments along the secretory, endocytic and degradative pathways. NHE6 may also be present on the plasmatic membrane [111] and plays an important role in the basolateral membrane of osteoblasts, particularly in regions of high mineralization, where it helps removing the excess of H^+ generated by this process. In addition, this transporter is also essential in bone turnover [142]. Its expression is notorious in hepatoma HepG2 cells, in which is present in recycling endosomes and co-localizes with transcytosing bulk membrane lipids. In this case, endosomal NHE6 is a key factor to maintain the polarized distribution of membrane lipids, the maintenance of apical bile canaliculi and consequently cell polarity [143]. NHE6 also plays an important role in the central nervous system. Functional studies showed that NHE6 KO mice present histological alterations in central nervous system that result in mild motor hyperactivity and deficits in motor coordination [144]. Moreover, these animals exhibit overacidified endosomes [145]. In humans, it is known that mutations in *SLC9A6* gene (codify NHE6 protein) can cause neurological disorders, such as the Angelman and the Christianson syndromes [146, 147].

NHE7 is an intracellular transporter that is mainly localized in the trans-Golgi network. This isoform mediates the influx of Na^+ or K^+ in exchange for H^+ [148] and plays an essential role in the control of cation homeostasis in this organelle (Figure 1.5a). NHE7 is expressed in high abundance in the stomach, pancreas, brain, prostate, skeletal muscle, and pituitary. It has been described that NHE7 has a role in cancer biology, but its physiology is not known yet [111, 149].

NHE8 is also mainly an intracellular transporter that is localized essentially on mid- to trans-Golgi network, but is also expressed in the apical membrane of renal proximal tubule and intestine [111, 150] (Figure 1.5a). It has been proposed that NHE8 may be recycled to the plasma membrane and be regulated by trafficking, like NHE3 [151]. Moreover, NHE3 and NHE8 are developmentally regulated. In the intestine, the expression of NHE8 is major in neonates, while NHE3 expression is more abundant in adults [150]. In fact, NHE8 KO mice do

not demonstrate alterations in blood pressure, due to a compensatory mechanism by NHE3 [152]. However, lack of NHE8 causes alterations in the intestine, decreasing mucus secretion and consequently increasing the susceptibility for gastric ulcers and bacterial infections [153, 154].

In what concerns to NHE9, this isoform is an endosomal cation/proton antiporter that alkalinize endosomal and vacuolar pH [155] (Figure 1.5a). It has been proposed that NHE9's function may be related to mental development, however there are not enough studies to prove its role in mammals [156].

SLC9Bs and SLC9Cs

SLC9Bs subgroup is constituted by two members, NHA1 and NHA2 (or also called NHEDC1 and NHDC2, respectively) [111]. Until now, the biological function of NHA1 remains unknown and its expression has not been related to any human disease. In relation to NHA2, some studies have produced some inconclusive results. NHA2 resides at the apical membrane of certain epithelial cells, as well as in that of endosomes [157, 158]. It has been proposed that NHA2 acts as a Na^+/Li^+ countertransporter, which had been related to arterial hypertension and diabetes [159, 160] (Figure 1.5a). This isoform is expressed in the kidney, where is important for the regulation of Na^+ and blood pressure homeostasis [161]. The NHA2 is also important in pancreas, where it was found in endosomes and synaptic-like microvesicles in β cells [162]. The loss of NHA2 affects insulin secretion indirectly by interfering with clathrin-mediated endocytosis in those cells [157, 163].

SLC9C subgroup is also constituted by two members, sNHE (SLC9C1) and NHE11 (SLC9C2). There are no functional data on NHE11, however, sNHE is a sperm-specific NHE (Figure 1.5a). sNHE is only present in male germ cells, and its expression is essential for male fertility [11, 164], as further discussed below.

Na^+/H^+ exchangers in male reproductive tract

As referred, the control of pH along the male reproductive tract is essential to spermatogenesis and sperm maturation. Indeed, alterations of pH equilibrium in male reproductive tract are associated with male infertility/subfertility in mammals [7]. The NHE family contributes to the acid-base homeostasis of several luminal fluids and various members of the NHE family have been described in the cells of the male reproductive tract, including the NHE1, NHE2, NHE3, NHE8 and sNHE. Among these isoforms, only NHE3, NHE8, and sNHE are reported to be directly associated with the establishment of male reproductive potential (Figure 1.2).

NHE1 was detected all along the male reproductive tract. In rats, NHE1 was localized on the basal surface of the tubular cells in the epididymis, which were more prominently expressed in

the corpus and the cauda, as well as in the vas deferens and efferent ducts [165, 166]. NHE1 is also expressed in the spermatozoa midpiece, where it can control the local concentration of intracellular H^+ concentration [167]. No other specific role has been described for NHE1 in the male reproductive tract, however, this transporter is characterized by controlling numerous functions in other systems. NHE1 controls intracellular pH, regulation of cell volume, cell proliferation and may also be implicated in transepithelial electrolyte transport [168].

NHE2 is another isoform that is expressed along the male reproductive tract but its function is still not well defined. NHE2 is expressed in the caput, corpus, and cauda of the epididymis, as well as in the efferent ducts and in testis [165, 169] (Figure 1.2).

NHE3 is expressed on the apical membrane of epithelial cells in the efferent duct and epididymis (Figure 1.2). It is known that this isoform has an essential role in fluid reabsorption in efferent ducts and pH control of epididymal fluid [166]. In fact, more than 96% of the fluid produced in the testis is reabsorbed by the efferent ducts. The fluid reabsorption is Na^+-dependent and significantly decreases when NHEs are blocked, suggesting that Na^+ reabsorption in efferent ducts is dependent of the NHEs [51]. Indeed, NHE3 KO mice showed tubular fluid accumulation and greatly dilated efferent ducts, which was associated with reduced fertility [170]. Zhou and collaborators [170], reported that ERα is responsible for the regulation of the expression of NHE3 in the efferent ducts and, thus, it influences Na^+ reabsorption and passive water transport. ERα KO mice and antiestrogen-treated mice presented loss or decreased expression of NHE3, which can be related with fluid accumulation in efferent ducts and testes in these animals, resulting in infertility [170]. Furthermore, NHE3 is also expressed in testicular cells, particularly in SCs [171]. However, the function of NHE3 on the seminiferous tubules and SCs has not been elucidated.

NHE8 was detected in human and mice testis, specifically in Leydig cells (Figure 1.2). Interestingly, NHE8 KO mice showed smaller testes and lacked spermatozoa in the seminiferous tubules and in the epididymis. Those animals were infertile, which led the authors to propose that it was partially due to the detected disorder in the expression of the luteinizing hormone (LH) and a consequent decreased testosterone production by Leydig cells, which led to morphological alterations in Sertoli cell [172].

Finally, sNHE is the sperm specific member of the mammalian NHE family. sNHE is expressed in the caudal epididymal spermatozoa and was detected in the principal piece of the sperm flagellum [167, 173] (Figure 1.2). Disruption of the *sNHE* gene in mice causes total male infertility associated with absolute loss of sperm motility [164]. The lack of sNHE resulted in a complete loss of full-length HCO_3^--sensitive soluble adenylate cyclase (sAC) with reduced sAC activity. sNHE and sAC physically interact in spermatozoa, which results in a signalling

complex at the sperm flagellar membrane that is of great importance to the intracellular HCO_3^- concentration and cAMP levels, both essentials to sperm motility and capacitation [164, 173].

Monocarboxylate transporters

The SLC16 family, more commonly refereed as monocarboxylate transporters (MCTs), are characterized by the transport across the plasma membrane of monocarboxylates, like lactate, pyruvate, branched-chain oxoacids, and ketone bodies, usually together with an H^+ [174]. However, transport preferences differs between MCT isoforms and not all MCTs transport monocarboxylates, as for instance MCT8, which transports the thyroid hormone [175]. MCTs play an important physiological role, since monocarboxylates are an important energy source for cells [176]. In addition to contributing to cellular metabolism, these transporters also play an important role to maintain a balance between intra- and extracellular pH. For instance, the transport of lactic acid (or lactate ion plus H^+), which is one of the end products of glycolysis, is performed essentially by MCTs [174, 177, 178]. Without its extrusion, an overload of lactic acid can occur, that would contribute to acidosis, a common feature of some pathologic conditions [179].

MCTs are ubiquitously distributed among several tissues. Fourteen members constitute the MCT family in mammals, however just seven members have been functionally characterized (MCT1-MCT4, MCT6, MCT8 and MCT10). Of all MCTs, only four members are proton-coupled (MCT1-MCT4) (Figure 1.5b). Hence, the following topic will focus only on these members due to the close relationship to pH control.

Proton-coupled monocarboxylate transporters

MCT1 is a membrane transporter that in epithelial cells can be present either in apical or basolateral membranes depending on the tissue. MCT1 has a great affinity for lactic acid, being that the major physiological function of this isoform is to facilitate L-lactic acid influx or efflux out of the cells depending on their metabolic status [177] (Figure 1.5b). The transport performed by MCT1 is regulated by its association with cell surface glycoprotein CD147, that has a transmembrane domain with the C-terminus in the cytosol [180]. However, the modulation of the activity of this protein appears to be regulated in each cell or in a tissue-specific manner [181, 182]. MCT1 catalyses either net transport of lactate with one H^+, or the exchange of one carboxylate for another. The transport is performed by a protein conformational change that translocate the substrate across the membrane, where the translocation cycle is reversible. The transport can be stimulated by decreasing or raising the pH inside or outside the cell [183, 184]. In cells with high glycolic fluxes (e.g. under hypoxia or anoxia) an intracellular acidification can occur due to high production of lactic acid by

glycolysis. In this metabolic condition, MCT1 is able to transport lactate and H^+. Therefore, MCT1 plays an essential role in lactate plus H^+ efflux and in the restoration of intracellular pH [176, 185]. In fact, lactic acid transport is performed by cooperation between the MCTs isoforms in several cells of the same tissue. Thus, these transporters work together to enable the equilibrium between influx and efflux of lactic acid [176].

MCT2 expression is more restrict than MCT1 and it has been observed that its localization varies among different species. Like MCT1, MCT2 has high affinity for lactic acid. But contrarily to MCT1, MCT2 has higher affinity to pyruvate in human cells [186, 187] (Figure 1.5b). The expression of MCT2 is mainly found in tissues that take up high quantities of lactic acid to use as a source of energy for cellular respiration (e.g., neurons) or for gluconeogenesis (e.g., liver cells) [176, 186]. MCT2 also requires an accessory protein for translocation to the plasma membrane, the gp70 (EMBIGIN), which allows the transport of lactate or pyruvate and H^+ [188].

MCT3 has the most restricted distribution of any MCT isoform, with its expression being essentially detected in the basal membrane of retinal pigment epithelium and choroid plexus of humans, rodents and chickens [189-192]. Concerning its functionality and physiological roles, the information about this isoform is still scarce. Nevertheless, decreased expression of MCT3 in smooth muscle cells has been associated with increasing severity of atherosclerosis [193], but the mechanism that triggers this association is not completely understood.

MCT4 is the last isoform proton-couplet MCT, and it demonstrates notable analogy to MCT1 relatively to tissue distribution, regulation, and substrate specificity. Despite a high overlap in specificity for substrates between MCT1 and MCT4, the last presents lower affinity for lactate and pyruvate [194] (Figure 1.5b). Like MCT1, MCT4 is localized in the plasma membrane and is dependent on glycoprotein CD147 to facilitate the substrate transport [195]. MCT4 is strongly expressed in tissues that rely on high levels of glycolysis to meet their energy requirements [174]. This isoform essentially participates in lactate efflux [178], as observed in cancer cells, muscle tissue or placenta. Cancer cells proliferation is dependent on anaerobic glycolysis that causes a higher consumption of glucose and an accumulation of glycolytic products, such as lactate. MCTs are key players for lactate export an essential process for the maintenance of the hyperglycolytic environment and for pH control, thus contributing to the acid-resistant phenotype [196]. In fact, some antitumor approaches are focused in the inhibition of these pH regulators for intra- and extracellular pH return to physiological levels, with the consequent impairment of tumour growth [197]. The role of MCT4 is also of great importance for muscular fibres, since its affinity for pyruvate is low. This kinetic characteristic ensures that pyruvate is not exported from the cells, which is essential for cells that are dependent on glycolysis: with the accumulation of pyruvate, its reduction to lactate and the production of NAD^+ is possible [176, 178]. On the other hand, MCT4 is also essential to prevent lactic acidosis during physical

exercise, due to the increased production of lactic acid and subsequent pH decrease [176, 178]. MCT4 has also a strong expression in placenta, which exports lactic acid from the foetal to the maternal circulation, preventing acidosis [198].

MCTs are also reported to cooperate with CAs to maintain pH and ionic balance. Although the details of how the regulation is done are not known, it has been described that MCT1 and MCT4 interact with CA II (intracellular isoform), and this interaction stimulates the transport by MCTs [199]. Moreover, it has been reported that the transport performed by MCT2 is reinforced by interaction with the CA IV (extracellular isoform) [200].

Proton-coupled monocarboxylates transporters in male reproductive tract

Some isoforms of proton-coupled MCTs play a particularly important role in the male reproductive tract. Starting from the testis, lactate plays an essential role in spermatogenesis and there will not be lactate transport without the expression and function of MCTs. SCs produce lactate to supply germ cells, particularly spermatocytes and spermatids that are unable to metabolize glucose [6]. Being so, the mechanisms that control lactate production and transport in SCs are extremely important to the maintenance of spermatogenesis and male fertility. MCT4 is the key transporter responsible for exporting lactate from the SCs to germ cells via MCT1 and MCT2 [6, 174, 201]. In the case of MCT1, its expression is rather controversial in SCs. This transporter has already been identified in rat SCs [202] and occasionally described in mouse SCs [203], but other studies argue that MCT1 is not expressed in these cells [204]. Nevertheless, the expression of MCT1 is well reported in spermatogonia, spermatocytes and spermatids, while MCT2 is restricted to the tails of elongated spermatids and spermatozoa [204]. Still, in the testis, it has been described that MCT1 is also expressed not only in Leydig cells, but also in efferent ducts and epididymis [205] (Figure 1.2). In fact, all proton-coupled MCTs (MCT1-MCT4) were detected in efferent ducts and epididymis of rat, with alteration in the expression during different phases of development [206, 207].

It is well known that the endocrine system plays a crucial role in the physiology of mammalian testes and in all reproductive tissues. Concerning MCTs, it has been described that ERα is able to mediate the regulation of MCT1 in rat efferent ducts, since in the absence of this receptor the expression of MCT1 significantly decreases [206]. Although the mechanism involved in this regulation is not yet known, it was proposed that MCTs might be involved in the regulation of fluid reabsorption, since s a great volume of testicular fluid is reabsorbed in the efferent duct [51]. Moreover, SCs are strongly regulated by steroid hormones, and the MCTs present in these cells are also modulated by those hormones. For instance, testosterone and its metabolite 5α-dihydrotestosterone (5α-DHT) modulate SCs metabolism. When these

cells are exposed to high levels of 5α-DHT, the lactate transported *via* MCT4 is decreased [208]. Additionally, high levels of testosterone and follicle-stimulating hormone (FSH) also exerted an negative effect on testicular MCT2 levels [209]. Human and rat SCs also showed an alteration in *MCT4* gene transcript levels after treatment with high levels of 17β-estradiol [201, 208]. Moreover, in insulin-deprivation conditions such as in the case of Diabetes mellitus, human SCs presented a decreased MCT4 expression and significantly lower production of lactate [210].

In sum, the proton-coupled MCTs seem to play essential roles in the establishment of a normal reproductive process. In addition to exert a high level of metabolic control, particularly in the testes, they also play an important role in pH control and fluid balance.

Concluding remarks

Spermatogenesis is a complex process that results in the production of viable and healthy spermatozoa. In normal conditions, these haploid cells should be capable of fertilizing the female gamete and originate a viable embryo. For this to occur, the establishment of an adequate environment throughout the male reproductive tract (with varying optimal pHs) is crucial. If this fundamental ionic (particularly the luminal fluids pHs) and metabolic balance established throughout the male reproductive tract is disturbed it can lead to changes in spermatozoa production. There are scarce studies that determine the composition of the fluids in the male reproductive tract and the transporter proteins that contribute to the ionic balance, due to the difficulty of the techniques for these evaluations, it is of extreme interest understand the composition and how the production of these fluids is regulated. The composition of the fluids throughout the male reproductive tract is extremely important for the formation, maturation and storage of spermatozoa. The proper transport of ions and metabolites to maintain adequate excurrent ducts luminal milieu is performed by several membrane proteins. It is clear that, as it happens in other tissues, the various membrane proteins already identified in the male reproductive tract that transport HCO_3^-, H^+, Na^+ and other ions, have at least one major function: maintain both intra- and extracellular pH within narrow limits allowing the reproductive process to occur and thus determining the reproductive potential of the individuals. It is known that ionic transport, in particular HCO_3^- and H^+, is of extreme importance for pH balance of the male reproductive tract fluids. This regulation is essential for the production of viable and fertilizable spermatozoa. Therefore, it is of great importance to unveil the exact role of the various HCO_3^- and H^+ membrane transporters present throughout the male reproductive tract and their contribution for the establishment of the luminal fluids. Further knowledge of the biochemical mechanisms that regulate ionic production and transport and

the influence of whole-body ionic status on reproduction is crucial, as well as on how these players contribute to the regulation of male reproductive potential.

References

1. Rowe, P.J., et al., WHO manual for the standardized investigation and diagnosis of the infertile male. 2000, Cambridge University Press, p. 1-87.
2. Brugh III, V.M. and L.I. Lipshultz, 2004, Male factor infertility: evaluation and management. Medical Clinics of North America, 88(2): p. 367-385.
3. Gannon, J.R. and T.J. Walsh, The epidemiology of male infertility, in Biennial Review of Infertility. 2015, Springer. p. 3-7.
4. Hamilton, B.E. and S.J. Ventura, 2006, Fertility and abortion rates in the United States, 1960–2002. International journal of andrology, 29(1): p. 34-45.
5. Lutz, W., 2006, Fertility rates and future population trends: will Europe's birth rate recover or continue to decline? International Journal of Andrology, 29(1): p. 25-33.
6. Rato, L., et al., 2012, Metabolic regulation is important for spermatogenesis. Nature Reviews Urology, 9(6): p. 330-8.
7. Pastor-Soler, N., C. Piétrement, and S. Breton, 2005, Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. Physiology, 20(6): p. 417-428.
8. Bernardino, R.L., Formation and biochemistry of seminal plasma and male accessory fluids, in Andrology: Current and Future Developments, M.G. Alves and P.F. Oliveira, Editors. 2017, Bentham Science Publishers, p. 207-229.
9. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. The Journal of membrane biology, 236(2): p. 215-224.
10. Bernardino, R.L., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current Medicinal Chemistry, 20(32): p. 4037-49.
11. Martins, A.D., et al., 2014, Physiology of na+/h+ exchangers in the male reproductive tract: relevance for male fertility. Biology of reproduction, 91(1): p. 11, 1-6.
12. Mann, T. and C. Lutwak-Mann, Testis and testicular semen, in Male Reproductive Function and Semen. 1981, Springer. p. 83-138.
13. Levine, N. and D.J. Marsh, 1971, Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. The Journal of physiology, 213(3): p. 557-570.
14. Levine, N. and H. Kelly, 1978, Measurement of pH in the rat epididymis in vivo. Journal of reproduction and fertility, 52(2): p. 333-335.

15. Rodríguez, C.M. and B.T. Hinton, The testicular and epididymal luminal fluid microenvironment, in Introduction to Mammalian Reproduction. 2003, Springer. p. 61-77.
16. Tao, L., J. Zupp, and B. Setchell, 2000, Effect of efferent duct ligation on the function of the blood-testis barrier in rats. Journal of Reproduction and Fertility, 120(1): p. 13-18.
17. Guyonnet, B., et al., 2011, The epididymal transcriptome and proteome provide some insights into new epididymal regulations. Journal of Andrology, 32(6): p. 651-64.
18. Marengo, S.R., 2008, Maturing the sperm: unique mechanisms for modifying integral proteins in the sperm plasma membrane. Animal Reproduction Science, 105(1): p. 52-63.
19. Elzanaty, S., et al., 2002, The impact of epididymal and accessory sex gland function on sperm motility. Human Reproduction, 17(11): p. 2904-2911.
20. Turner, T., Necessity's potion: inorganic ions and small organic molecules in the epididymal lumen, in The Epididymis: From Molecules to Clinical Practice. 2002, Springer. p. 131-150.
21. Robaire, B., B.T. Hinton, and M.C. Orgebin-Crist, The epididymis, in The Physiology of reproduction, T. Plant and A. Zeleznik, Editors. 2006, Elsevier, p. 1071-1148.
22. Kirchhoff, C., et al., 1998, Function of human epididymal proteins in sperm maturation. Andrologia, 30(4-5): p. 225-32.
23. Cornwall, G.A., 2009, New insights into epididymal biology and function. Human Reproduction Update, 15(2): p. 213-27.
24. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. Journal of Membrane Biology, 236(2): p. 215-24.
25. Asari, M., et al., 1996, Immunohistolocalization of the carbonic anhydrase isoenzymes (CA-I, CA-II, and CA-III) in the reproductive tract of male horses. American Journal of Veterinary Research, 57(4): p. 439-443.
26. Zippin, J.H., et al., 2003, Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. The FASEB Journal, 17(1): p. 82-84.
27. Dacheux, J.-L. and F. Dacheux, 2014, New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2): p. R27-R42.
28. Chen, Y., et al., 2000, Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science, 289(5479): p. 625-628.
29. Vishwakarma, P., 1962, The pH and bicarbonate-ion content of the oviduct and uterine fluids. Fertility and sterility, 13(5): p. 481-485.
30. Swietach, P., R.D. Vaughan-Jones, and A.L. Harris, 2007, Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer and Metastasis Reviews, 26(2): p. 299-310.
31. Meldrum, N.U. and F.J.W. Roughton, 1933, Carbonic anhydrase. Its preparation and properties. The Journal of physiology, 80(2): p. 113-142.

32. Jacobs, M. and D.R. Stewart, 1942, The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. The Journal of general physiology, 25(4): p. 539-552.
33. Cohen, J.P., A.P. Hoffer, and S. Rosen, 1976, Carbonic anhydrase localization in the epididymis and testis of the rat: histochemical and biochemical analysis. Biology of Reproduction, 14(3): p. 339-346.
34. Caflisch, C.R. and T. DuBose Jr, 1990, Direct evaluation of acidification by rat testis and epididymis: role of carbonic anhydrase. American Journal of Physiology-Endocrinology And Metabolism, 258(1): p. E143-E150.
35. Breton, S., 2001, The cellular physiology of carbonic anhydrases. Journal of Pancreas, 2(4 Suppl): p. 159-164.
36. Hewett-Emmett, D., Evolution and distribution of the carbonic anhydrase gene families, in The Carbonic Anhydrases. 2000, Springer. p. 29-76.
37. Supuran, C.T. and A. Scozzafava, 2000, Carbonic anhydrase inhibitors and their therapeutic potential. Expert Opinion on Therapeutic Patents, 10(5): p. 575-600.
38. Nishimori, I., et al., 2007, Carbonic anhydrase inhibitors. DNA cloning, characterization, and inhibition studies of the human secretory isoform VI, a new target for sulfonamide and sulfamate inhibitors. Journal of medicinal chemistry, 50(2): p. 381-388.
39. Liu, Y., D.-K. Wang, and L.-M. Chen, 2012, The physiology of bicarbonate transporters in mammalian reproduction. Biology of reproduction, 86(4): p. 99, 1-13.
40. Setchell, B. and B. Brown, 1972, The effect of metabolic alkalosis, hypotension and inhibitors of carbonic anhydrase on fluid secretion by rat testes. Journal of reproduction and fertility, 28(2): p. 235-240.
41. Ekstedt, E., L. Holm, and Y. Ridderstråle, 2004, Carbonic anhydrase in mouse testis and epididymis; transfer of isozyme IV to spermatozoa during passage. Journal of molecular histology, 35(2): p. 167-173.
42. Waheed, A., X. Zhu, and W. Sly, 1992, Membrane-associated carbonic anhydrase from rat lung. Purification, characterization, tissue distribution, and comparison with carbonic anhydrase IVs of other mammals. Journal of Biological Chemistry, 267(5): p. 3308-3311.
43. Parkkila, S., et al., 1991, A high activity carbonic anhydrase isoenzyme (CA II) is present in mammalian spermatozoa. Histochemistry and Cell Biology, 95(5): p. 477-482.
44. Acott, T.S. and D.W. Carr, 1984, Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. Biology of reproduction, 30(4): p. 926-935.
45. Okamura, N., et al., 1986, Lowered levels of bicarbonate in seminal plasma cause the poor sperm motility in human infertile patients. Fertility and sterility, 45(2): p. 265-272.

46. Okamura, N., et al., 1985, Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. Journal of Biological Chemistry, 260(17): p. 9699-9705.
47. Au, C. and P. Wong, 1980, Luminal acidification by the perfused rat cauda epididymidis. The Journal of physiology, 309(1): p. 419-427.
48. Harris, G. and K. Goto, 1984, Carbonic anhydrase activity of the reproductive tract tissues of aged male fowls and its relationship to semen production. Journal of reproduction and fertility, 70(1): p. 25-30.
49. Hermo, L., et al., 2005, Region-and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. Journal of Histochemistry & Cytochemistry, 53(6): p. 699-713.
50. Kaunisto, K., et al., 1995, Expression of carbonic anhydrase isoenzymes IV and II in rat epididymal duct. Biology of reproduction, 52(6): p. 1350-1357.
51. Clulow, J., R. Jones, and L. Hansen, 1994, Micropuncture and cannulation studies of fluid composition and transport in the ductuli efferentes testis of the rat: comparisons with the homologous metanephric proximal tubule. Experimental Physiology, 79(6): p. 915-928.
52. Karhumaa, P., et al., 2001, Expression of the transmembrane carbonic anhydrases, CA IX and CA XII, in the human male excurrent ducts. Molecular human reproduction, 7(7): p. 611-616.
53. Jensen, L.J., et al., 1999, Immunolocalization of AE2 anion exchanger in rat and mouse epididymis. Biology of reproduction, 61(4): p. 973-980.
54. Ivanov, S., et al., 2001, Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. The American journal of pathology, 158(3): p. 905-919.
55. Van Slyke, D.D. and G.E. Cullen, 1917, Studies of acidosis I. The bicarbonate concentration of the blood plasma; its significance, and its determination as a measure of acidosis. Journal of Biological Chemistry, 30(2): p. 289-346.
56. Tresguerres, M., J. Buck, and L.R. Levin, 2010, Physiological carbon dioxide, bicarbonate, and pH sensing. Pflügers Archiv-European Journal of Physiology, 460(6): p. 953-964.
57. Gluck, S.L., 2004, Acid sensing in renal epithelial cells. The Journal of clinical investigation, 114(12): p. 1696-1699.
58. Alper, S.L., 2009, Molecular physiology and genetics of Na+-independent SLC4 anion exchangers. Journal of Experimental Biology, 212(11): p. 1672-1683.
59. Sindic, A., et al., 2007, Renal physiology of SLC26 anion exchangers. Current opinion in nephrology and hypertension, 16(5): p. 484-490.

60. Ishiguro, H., et al., 2009, CFTR functions as a bicarbonate channel in pancreatic duct cells. The Journal of general physiology, 133(3): p. 315-326.
61. Bernardino, R.L., et al., 2013, Effect of prediabetes on membrane bicarbonate transporters in testis and epididymis. The Journal of membrane biology, 246(12): p. 877-883.
62. Fujinaga, J., F.B. Loiselle, and J.R. Casey, 2003, Transport activity of chimaeric AE2-AE3 chloride/bicarbonate anion exchange proteins. Biochemical Journal, 371(Pt 3): p. 687.
63. Passow, H., Molecular aspects of band 3 protein-mediated anion transport across the red blood cell membrane, in Reviews of Physiology, Biochemistry and Pharmacology, Volume 103. 1986, Springer. p. 61-203.
64. Humphreys, B., et al., 1995, Hypertonic activation of AE2 anion exchanger in Xenopus oocytes via NHE-mediated intracellular alkalinization. American Journal of Physiology-Cell Physiology, 268(1): p. C201-C209.
65. Zhang, Y., et al., 1996, The cytoplasmic and transmembrane domains of AE2 both contribute to regulation of anion exchange by pH. Journal of Biological Chemistry, 271(10): p. 5741-5749.
66. Stewart, A., et al., 2001, Regulation of AE2 anion exchanger by intracellular pH: critical regions of the NH2-terminal cytoplasmic domain. American Journal of Physiology-Cell Physiology, 281(4): p. C1344-C1354.
67. Casey, J.R., S. Grinstein, and J. Orlowski, 2010, Sensors and regulators of intracellular pH. Nature reviews Molecular cell biology, 11(1): p. 50.
68. Romero, M.F., C.M. Fulton, and W.F. Boron, 2004, The SLC4 family of HCO3− transporters. Pflügers Archiv, 447(5): p. 495-509.
69. Marino, C.R., et al., 1999, Expression and distribution of the Na+-HCO 3− cotransporter in human pancreas. American Journal of Physiology-Gastrointestinal and Liver Physiology, 277(2): p. G487-G494.
70. Burnham, C.E., et al., 1997, Cloning and functional expression of a human kidney Na+: HCO3− cotransporter. Journal of Biological Chemistry, 272(31): p. 19111-19114.
71. Boron, W.F. and E.L. Boulpaep, 1989, The electrogenic Na/HCO3 cotransporter. Kidney international, 36(3): p. 392-402.
72. Boron, W.F., 2001, Sodium-coupled bicarbonate transporters. Jop, 2(4 Suppl): p. 176-181.
73. Russell, J.M. and W.F. Boron, 1976, Role of chloride transport in regulation of intracellular pH. Nature, 264(5581): p. 73.
74. Grichtchenko, I.I., et al., 2001, Cloning, Characterization, and Chromosomal Mapping of a Human Electroneutral Na+-driven Cl-HCO3Exchanger. Journal of Biological Chemistry, 276(11): p. 8358-8363.

75. Parker, M.D., et al., 2008, Characterization of human SLC4A10 as an electroneutral Na/HCO3 cotransporter (NBCn2) with Cl–self-exchange activity. Journal of Biological Chemistry, 283(19): p. 12777-12788.
76. Karniski, L.P., et al., 1998, Immunolocalization of sat-1 sulfate/oxalate/bicarbonate anion exchanger in the rat kidney. American Journal of Physiology-Renal Physiology, 275(1): p. F79-F87.
77. Mount, D.B. and M.F. Romero, 2004, The SLC26 gene family of multifunctional anion exchangers. Pflügers Archiv : European journal of physiology, 447(5): p. 710-721.
78. Ko, S.B., et al., 2002, A molecular mechanism for aberrantCFTR-dependent HCO3− transport in cystic fibrosis. The EMBO journal, 21(21): p. 5662-5672.
79. Shcheynikov, N., et al., 2006, Coupling modes and stoichiometry of Cl−/HCO3− exchange by slc26a3 and slc26a6. The Journal of general physiology, 127(5): p. 511-524.
80. Schweinfest, C.W., et al., 1993, Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas. Proceedings of the National Academy of Sciences, 90(9): p. 4166-4170.
81. Simpson, J.E., et al., 2007, PAT-1 (Slc26a6) is the predominant apical membrane Cl−/HCO3− exchanger in the upper villous epithelium of the murine duodenum. American Journal of Physiology-Gastrointestinal and Liver Physiology, 292(4): p. G1079-G1088.
82. Wang, Y., et al., 2006, Slc26a6 regulates CFTR activity in vivo to determine pancreatic duct HCO3− secretion: relevance to cystic fibrosis. The EMBO journal, 25(21): p. 5049-5057.
83. Kim, K.H., et al., 2005, SLC26A7 is a Cl–channel regulated by intracellular pH. Journal of Biological Chemistry, 280(8): p. 6463-6470.
84. Lohi, H., et al., 2002, Functional characterization of three novel tissue-specific anion exchangers SLC26A7,-A8, and-A9. Journal of Biological Chemistry, 277(16): p. 14246-14254.
85. Ousingsawat, J., R. Schreiber, and K. Kunzelmann, 2012, Differential contribution of SLC26A9 to Cl− conductance in polarized and non-polarized epithelial cells. Journal of cellular physiology, 227(6): p. 2323-2329.
86. Avella, M., et al., 2011, SLC26A9 stimulates CFTR expression and function in human bronchial cell lines. Journal of cellular physiology, 226(1): p. 212-223.
87. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and Cellular Biochemistry, 408(1-2): p. 47-54.
88. Holappa, K., et al., 1999, Primary structure of a sperm cell anion exchanger and its messenger ribonucleic acid expression during spermatogenesis. Biology of reproduction, 61(4): p. 981-986.

89. Chen, L.M., Y. Liu, and W.F. Boron, 2011, Role of an extracellular loop in determining the stoichiometry of Na+–HCO3− cotransporters. The Journal of physiology, 589(4): p. 877-890.
90. Medina, J.F., et al., 2003, Anion exchanger 2 is essential for spermiogenesis in mice. Proceedings of the National Academy of Sciences, 100(26): p. 15847-15852.
91. Uhlen, M., et al., 2010, Towards a knowledge-based human protein atlas. Nature biotechnology, 28(12): p. 1248-1250.
92. Jensen, L.J., et al., 1999, Localization of sodium bicarbonate cotransporter (NBC) protein and messenger ribonucleic acid in rat epididymis. Biology of reproduction, 60(3): p. 573-579.
93. Liu, Y., et al., 2011, Cloning and identification of two novel NBCe1 splice variants from mouse reproductive tract tissues: a comparative study of NCBT genes. Genomics, 98(2): p. 112-119.
94. Nishimura, M. and S. Naito, 2005, Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies. Drug metabolism and pharmacokinetics, 20(6): p. 452-477.
95. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biologi of the Cell, 108(7): p. 179-188.
96. Chan, H., et al., 1996, Evidence for independent Cl-and HCO3-secretion and involvement of an apical Na (+)-HCO3-cotransporter in cultured rat epididymal epithelia. Experimental physiology, 81(3): p. 515-524.
97. Wang, C.-Z., et al., 2000, The Na+-driven Cl−/HCO3− Exchanger Cloning, Tissue Distribution, and Functional Characterization. Journal of Biological Chemistry, 275(45): p. 35486-35490.
98. Joseph, A., et al., 2010, Absence of estrogen receptor alpha leads to physiological alterations in the mouse epididymis and consequent defects in sperm function. Biology of reproduction, 82(5): p. 948-957.
99. Pierucci-Alves, F., V. Akoyev, and B.D. Schultz, 2015, Bicarbonate exchangers SLC26A3 and SLC26A6 are localized at the apical membrane of porcine vas deferens epithelium. Physiological reports, 3(4), p. e12380.
100. Dirami, T., et al., 2013, Missense mutations in SLC26A8, encoding a sperm-specific activator of CFTR, are associated with human asthenozoospermia. The American Journal of Human Genetics, 92(5): p. 760-766.

101. Toure, A., et al., 2007, The testis anion transporter 1 (Slc26a8) is required for sperm terminal differentiation and male fertility in the mouse. Human molecular genetics, 16(15): p. 1783-1793.
102. Hernández-González, E.O., et al., 2007, Involvement of cystic fibrosis transmembrane conductance regulator in mouse sperm capacitation. Journal of Biological Chemistry, 282(33): p. 24397-24406.
103. Xu, W.M., et al., 2007, Cystic fibrosis transmembrane conductance regulator is vital to sperm fertilizing capacity and male fertility. Proceedings of the National Academy of Sciences, 104(23): p. 9816-9821.
104. Chávez, J.C., et al., 2012, Participation of the Cl−/HCO3− exchangers SLC26A3 and SLC26A6, the Cl− channel CFTR, and the regulatory factor SLC9A3R1 in mouse sperm capacitation. Biology of reproduction, 86(1): p. 1-14.
105. Hayashi, H., K. Suruga, and Y. Yamashita, 2009, Regulation of intestinal Cl−/HCO3− exchanger SLC26A3 by intracellular pH. American Journal of Physiology-Cell Physiology, 296(6): p. C1279-C1290.
106. Chen, W.Y., et al., 2009, Cl− is required for HCO3− entry necessary for sperm capacitation in guinea pig: involvement of a Cl−/HCO3− exchanger (SLC26A3) and CFTR. Biology of reproduction, 80(1): p. 115-123.
107. Holmberg, C., J. Perheentupa, and K. Launiala, 1975, Colonic electrolyte transport in health and in congenital chloride diarrhea. The Journal of clinical investigation, 56(2): p. 302-310.
108. Höglund, P., et al., 2006, Disruption of the SLC26A3-mediated anion transport is associated with male subfertility. Fertility and sterility, 85(1): p. 232-235.
109. Mahnensmith, R.L. and P.S. Aronson, 1985, The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. Circulation research, 56(6): p. 773-788.
110. Lacroix, J.m., et al., 2004, A mechanism for the activation of the Na/H exchanger NHE-1 by cytoplasmic acidification and mitogens. EMBO reports, 5(1): p. 91-96.
111. Fuster, D.G. and R.T. Alexander, 2014, Traditional and emerging roles for the SLC9 Na+/H+ exchangers. Pflügers Archiv-European Journal of Physiology, 466(1): p. 61-76.
112. Wakabayashi, S., M. Shigekawa, and J. Pouyssegur, 1997, Molecular physiology of vertebrate Na+/H+ exchangers. Physiological Reviews, 77(1): p. 51-74.
113. Haworth, R., O. Fröhlich, and L. Fliegel, 1993, Multiple carbohydrate moieties on the Na+/H+ exchanger. Biochemical Journal, 289(3): p. 637-640.
114. Grinstein, S., et al., 1993, Focal localization of the NHE-1 isoform of the Na+/H+ antiport: assessment of effects on intracellular pH. The EMBO Journal, 12(13): p. 5209-5218.

115. Shimoda, L.A., et al., 2006, HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. American Journal of Physiology-Lung Cellular and Molecular Physiology, 291(5): p. L941-L949.
116. Bell, S.M., et al., 1999, Targeted disruption of the murine Nhe1 locus induces ataxia, growth retardation, and seizures. American Journal of Physiology-Cell Physiology, 276(4): p. C788-C795.
117. Gu, X.Q., H. Yao, and G.G. Haddad, 2001, Increased neuronal excitability and seizures in the Na+/H+ exchanger null mutant mouse. American Journal of Physiology-Cell Physiology, 281(2): p. C496-C503.
118. Xia, Y., et al., 2003, Na+ channel expression and neuronal function in the Na+/H+ exchanger 1 null mutant mouse. Journal of neurophysiology, 89(1): p. 229-236.
119. Yao, H., et al., 1999, Intracellular pH regulation of CA1 neurons in Na+/H+ isoform 1 mutant mice. The Journal of clinical investigation, 104(5): p. 637-645.
120. Villa-Abrille, M.C., et al., 2011, Silencing of cardiac mitochondrial NHE1 prevents mitochondrial permeability transition pore opening. American Journal of Physiology-Heart and Circulatory Physiology, 300(4): p. H1237-H1251.
121. Karmazyn, M., 1988, Amiloride enhances postischemic ventricular recovery: possible role of Na+-H+ exchange. American Journal of Physiology-Heart and Circulatory Physiology, 255(3): p. H608-H615.
122. Luo, J., et al., 2005, Decreased neuronal death in Na+/H+ exchanger isoform 1-null mice after in vitro and in vivo ischemia. Journal of Neuroscience, 25(49): p. 11256-11268.
123. Chambrey, R., et al., 1998, Immunolocalization of the Na+/H+ exchanger isoform NHE2 in rat kidney. American Journal of Physiology-Renal Physiology, 275(3): p. F379-F386.
124. Hoogerwerf, W.A., et al., 1996, NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. American Journal of Physiology-Gastrointestinal and Liver Physiology, 270(1): p. G29-G41.
125. Schultheis, P.J., et al., 1998, Targeted disruption of the murine Na+/H+ exchanger isoform 2 gene causes reduced viability of gastric parietal cells and loss of net acid secretion. The Journal of clinical investigation, 101(6): p. 1243-1253.
126. Miller, M.L., et al., 2011, Loss of the NHE2 Na+/H+ Exchanger in Mice Results in Dilation of Folliculo-Stellate Cell Canaliculi. BioMed Research International, 2011: p. 1-14.
127. D'Souza, S., et al., 1998, The epithelial sodium-hydrogen antiporter Na+/H+ exchanger 3 accumulates and is functional in recycling endosomes. Journal of Biological Chemistry, 273(4): p. 2035-2043.

128. Szászi, K., et al., 2002, Clathrin-mediated Endocytosis and Recycling of the Neuron-specific Na+/H+ Exchanger NHE5 Isoform regulation by phosphatidylinositol 3′-kinase and the actin cytoskeleton. Journal of Biological Chemistry, 277(45): p. 42623-42632.

129. Ledoussal, C., et al., 2001, Renal salt wasting in mice lacking NHE3 Na+/H+ exchanger but not in mice lacking NHE2. American Journal of Physiology-Renal Physiology, 281(4): p. F718-F727.

130. Wang, T., et al., 1999, Mechanism of proximal tubule bicarbonate absorption in NHE3 null mice. American Journal of Physiology-Renal Physiology, 277(2): p. F298-F302.

131. Li, H.C., et al., 2013, Proximal tubule specific knockout of the Na+/H+ exchanger NHE3: effects on bicarbonate absorption and ammonium excretion. Journal of Molecular Medicine, 91(8): p. 951-963.

132. Schultheis, P.J., et al., 1998, Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. Nature genetics, 19(3): p. 282.

133. Gekle, M., et al., 1999, Inhibition of Na+-H+ exchange impairs receptor-mediated albumin endocytosis in renal proximal tubule-derived epithelial cells from opossum. The Journal of physiology, 520(3): p. 709-721.

134. Weisz, O.A., 2003, Acidification and protein traffic. International review of cytology, 226: p. 259-320.

135. Gekle, M., et al., 2004, NHE3 Na+/H+ exchanger supports proximal tubular protein reabsorption in vivo. American Journal of Physiology-Renal Physiology, 287(3): p. F469-F473.

136. Gawenis, L.R., et al., 2005, Impaired gastric acid secretion in mice with a targeted disruption of the NHE4 Na+/H+ exchanger. Journal of Biological Chemistry, 280(13): p. 12781-12789.

137. Bourgeois, S., et al., 2010, NHE4 is critical for the renal handling of ammonia in rodents. The Journal of clinical investigation, 120(6): p. 1895-1904.

138. Attaphitaya, S., K. Park, and J.E. Melvin, 1999, Molecular cloning and functional expression of a rat Na+/H+ exchanger (NHE5) highly expressed in brain. Journal of Biological Chemistry, 274(7): p. 4383-4388.

139. Klanke, C.A., et al., 1995, Molecular cloning and physical and genetic mapping of a novel human Na+/H+ exchanger (NHE5/SLC9A5) to chromosome 16q22. 1. Genomics, 25(3): p. 615-622.

140. Scott, C.C. and J. Gruenberg, 2011, Ion flux and the function of endosomes and lysosomes: pH is just the start. Bioessays, 33(2): p. 103-110.

141. Ohgaki, R., et al., 2010, Organellar Na+/H+ exchangers: novel players in organelle pH regulation and their emerging functions. Biochemistry, 50(4): p. 443-450.

142. Liu, L., et al., 2011, High capacity Na+/H+ exchange activity in mineralizing osteoblasts. Journal of cellular physiology, 226(6): p. 1702-1712.

143. Ohgaki, R., et al., 2010, The Na+/H+ exchanger NHE6 in the endosomal recycling system is involved in the development of apical bile canalicular surface domains in HepG2 cells. Molecular biology of the cell, 21(7): p. 1293-1304.

144. Strømme, P., et al., 2011, X-linked Angelman-like syndrome caused by Slc9a6 knockout in mice exhibits evidence of endosomal–lysosomal dysfunction. Brain, 134(11): p. 3369-3383.

145. Schwede, M., et al., 2014, Genes for endosomal NHE6 and NHE9 are misregulated in autism brains. Molecular psychiatry, 19(3): p. 277.

146. Christianson, A.L., et al., 1999, X linked severe mental retardation, craniofacial dysmorphology, epilepsy, ophthalmoplegia, and cerebellar atrophy in a large South African kindred is localised to Xq24-q27. Journal of medical genetics, 36(10): p. 759-766.

147. Gilfillan, G.D., et al., 2008, SLC9A6 mutations cause X-linked mental retardation, microcephaly, epilepsy, and ataxia, a phenotype mimicking Angelman syndrome. The American Journal of Human Genetics, 82(4): p. 1003-1010.

148. Numata, M. and J. Orlowski, 2001, Molecular cloning and characterization of a novel (Na+, K+)/H+ exchanger localized to the trans-Golgi network. Journal of Biological Chemistry, 276(20): p. 17387-17394.

149. Kagami, T., et al., 2008, Identification and biochemical characterization of the SLC9A7 interactome. Molecular membrane biology, 25(5): p. 436-447.

150. Becker, A.M., et al., 2007, Ontogeny of NHE8 in the rat proximal tubule. American Journal of Physiology-Renal Physiology, 293(1): p. F255-F261.

151. Bobulescu, I.A., F. Di Sole, and O.W. Moe, 2005, Na+/H+ exchangers: physiology and link to hypertension and organ ischemia. Current opinion in nephrology and hypertension, 14(5): p. 485.

152. Baum, M., et al., 2012, Proximal tubule Na+/H+ exchanger activity in adult NHE8−/−, NHE3−/−, and NHE3−/−/NHE8−/− mice. American Journal of Physiology-Renal Physiology, 303(11): p. F1495-F1502.

153. Liu, C., et al., 2013, NHE8 plays an important role in mucosal protection via its effect on bacterial adhesion. American Journal of Physiology-Cell Physiology, 305(1): p. C121-C128.

154. Xu, H., et al., 2012, NHE8 plays important roles in gastric mucosal protection. American Journal of Physiology-Gastrointestinal and Liver Physiology, 304(3): p. G257-G261.

155. Brett, C.L., et al., 2005, The yeast endosomal Na+ (K+)/H+ exchanger Nhx1 regulates cellular pH to control vesicle trafficking. Molecular biology of the cell, 16(3): p. 1396-1405.

156. Kondapalli, K.C., et al., 2013, Functional evaluation of autism-associated mutations in NHE9. Nature communications, 4: 2510.

157. Deisl, C., et al., 2013, Sodium/hydrogen exchanger NHA2 is critical for insulin secretion in β-cells. Proceedings of the National Academy of Sciences, 110(24): p. 10004-10009.

158. Hofstetter, W., et al., 2010, Sodium/hydrogen exchanger NHA2 in osteoclasts: subcellular localization and role in vitro and in vivo. Bone, 47(2): p. 331-340.

159. Canessa, M., et al., 1980, Increased sodium-lithium countertransport in red cells of patients with essential hypertension. New England Journal of Medicine, 302(14): p. 772-776.

160. Canessa, M., G. Zerbini, and L. Laffel, 1992, Sodium activation kinetics of red blood cell Na+/Li+ countertransport in diabetes: methodology and controversy. Journal of the American Society of Nephrology, 3(4): p. S41.

161. Fuster, D.G., et al., 2008, Characterization of the sodium/hydrogen exchanger NHA2. Journal of the American Society of Nephrology, 19(8): p. 1547-1556.

162. Orci, L., et al., 1973, Exocytosis-endocytosis coupling in the pancreatic beta cell. Science, 181(4099): p. 561-562.

163. Kimura, T., et al., 2008, The GDP-dependent Rab27a effector coronin 3 controls endocytosis of secretory membrane in insulin-secreting cell lines. Journal of Cell Science, 121(18): p. 3092-3098.

164. Wang, D., et al., 2007, A sperm-specific Na+/H+ exchanger (sNHE) is critical for expression and in vivo bicarbonate regulation of the soluble adenylyl cyclase (sAC). Proceedings of the National Academy of Sciences, 104(22): p. 9325-9330.

165. Chew, S.C., et al., 2000, Polarized distribution of NHE1 and NHE2 in the rat epididymis. Biology of reproduction, 62(3): p. 755-758.

166. Leung, G., et al., 2001, Expression of multiple Na+/H+ exchanger isoforms in cultured epithelial cells from rat efferent duct and cauda epididymidis. Biology of reproduction, 64(2): p. 482-490.

167. Woo, A.L., P.F. James, and J.B. Lingrel, 2002, Roles of the Na, K-ATPase α4 isoform and the Na+/H+ exchanger in sperm motility. Molecular reproduction and development, 62(3): p. 348-356.

168. Yun, C., et al., 1995, Mammalian Na+/H+ exchanger gene family: structure and function studies. American Journal of Physiology-Gastrointestinal and Liver Physiology, 269(1): p. G1-G11.

169. Malakooti, J., et al., 1999, Molecular cloning, tissue distribution, and functional expression of the human Na+/H+ exchanger NHE2. American Journal of Physiology-Gastrointestinal and Liver Physiology, 277(2): p. G383-G390.

170. Zhou, Q., et al., 2001, Estrogen action and male fertility: roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. Proceedings of the National Academy of Sciences of the United States of America, 98(24): p. 14132-7.
171. Gorczyńska-Fjälling, E., 2004, The role of calcium in signal transduction processes in Sertoli cells. Reprod Biol, 4: p. 219-241.
172. Xu, H., et al., 2014, Disruption of NHE8 expression impairs Leydig cell function in the testes. American Journal of Physiology-Cell Physiology, 308(4): p. C330-C338.
173. Wang, D., et al., 2003, A new sperm-specific Na+/H+ exchanger required for sperm motility and fertility. Nature cell biology, 5(12): p. 1117-1122.
174. Halestrap, A.P. and N.T. Price, 1999, The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochemical Journal, 343(2): p. 281-299.
175. Morris, M.E. and M.A. Felmlee, 2008, Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse γ-hydroxybutyric acid. The AAPS journal, 10(2): p. 311-321.
176. Halestrap, A.P. and D. Meredith, 2004, The SLC16 gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflügers Archiv : European journal of physiology, 447(5): p. 619-628.
177. Halestrap, A.P. and M.C. Wilson, 2012, The monocarboxylate transporter family—role and regulation. IUBMB life, 64(2): p. 109-119.
178. Juel, C. and A.P. Halestrap, 1999, Lactate transport in skeletal muscle—role and regulation of the monocarboxylate transporter. The Journal of physiology, 517(3): p. 633-642.
179. Brahimi-Horn, M.C., J. Chiche, and J. Pouyssegur, 2007, Hypoxia signalling controls metabolic demand. Current opinion in cell biology, 19(2): p. 223-229.
180. Kirk, á., et al., 2000, CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. The EMBO journal, 19(15): p. 3896-3904.
181. Cuff, M.A. and S.P. Shirazi-Beechey, 2002, The human monocarboxylate transporter, MCT1: genomic organization and promoter analysis. Biochemical and biophysical research communications, 292(4): p. 1048-1056.
182. Enoki, T., et al., 2006, Testosterone increases lactate transport, monocarboxylate transporter (MCT) 1 and MCT4 in rat skeletal muscle. The Journal of physiology, 577(1): p. 433-443.
183. Poole, R.C. and A.P. Halestrap, 1993, Transport of lactate and other monocarboxylates across mammalian plasma membranes. American Journal of Physiology-Cell Physiology, 264(4): p. C761-C782.

184. Deuticke, B., 1982, Monocarboxylate transport in erythrocytes. The Journal of membrane biology, 70(2): p. 89-103.

185. Halestrap, A.P., et al., 1997, Lactate transport in heart in relation to myocardial ischemia. American Journal of Cardiology, 80(3): p. 17A-25A.

186. Garcia, C.K., et al., 1995, cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1. Journal of Biological Chemistry, 270(4): p. 1843-1849.

187. Broer, S., et al., 1999, Characterization of the high-affinity monocarboxylate transporter MCT2 in Xenopus laevis oocytes. Biochemical Journal, 341(3): p. 529-535.

188. Wilson, M.C., et al., 2005, Basigin (CD147) Is the Target for Organomercurial Inhibition of Monocarboxylate Transporter Isoforms 1 and 4 the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70). Journal of Biological Chemistry, 280(29): p. 27213-27221.

189. Philp, N.J., H. Yoon, and L. Lombardi, 2001, Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. American Journal of Physiology-Cell Physiology, 280(5): p. C1319-C1326.

190. Yoon, H., et al., 1997, Identification of a unique monocarboxylate transporter (MCT3) in retinal pigment epithelium. Biochemical and biophysical research communications, 234(1): p. 90-94.

191. Philp, N.J., H. Yoon, and E.F. Grollman, 1998, Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 274(6): p. R1824-R1828.

192. Grollman, E.F., et al., 2000, Determination of transport kinetics of chick MCT3 monocarboxylate transporter from retinal pigment epithelium by expression in genetically modified yeast. Biochemistry, 39(31): p. 9351-9357.

193. Zhu, S., P.J. Goldschmidt-Clermont, and C. Dong, 2005, Inactivation of monocarboxylate transporter MCT3 by DNA methylation in atherosclerosis. Circulation, 112(9): p. 1353-1361.

194. Fox, J.E.M., D. Meredith, and A.P. Halestrap, 2000, Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. The Journal of physiology, 529(2): p. 285-293.

195. Gallagher, S.M., et al., 2007, Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. Cancer research, 67(9): p. 4182-4189.

196. Hanahan, D. and R.A. Weinberg, 2011, Hallmarks of cancer: the next generation. Cell, 144(5): p. 646-674.

197. Neri, D. and C.T. Supuran, 2011, Interfering with pH regulation in tumours as a therapeutic strategy. Nature reviews Drug discovery, 10(10): p. 767-777.

198. Price, T.N., N.V. Jackson, and P.A. Halestrap, 1998, Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. Biochemical Journal, 329(2): p. 321-328.

199. Becker, H.M., et al., 2011, Intramolecular proton shuttle supports not only catalytic but also noncatalytic function of carbonic anhydrase II. Proceedings of the National Academy of Sciences, 108(7): p. 3071-3076.

200. Klier, M., et al., 2011, Transport activity of the high-affinity monocarboxylate transporter MCT2 is enhanced by extracellular carbonic anhydrase IV but not by intracellular carbonic anhydrase II. Journal of Biological Chemistry, 286(31): p. 27781-27791.

201. Oliveira, P., et al., 2011, Influence of 5α-dihydrotestosterone and 17β-estradiol on human Sertoli cells metabolism. International journal of andrology, 34(6pt2).

202. Galardo, M.N., et al., 2007, The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-bD-ribonucleoside, regulates lactate production in rat Sertoli cells. Journal of molecular endocrinology, 39(4): p. 279-288.

203. Chen, C., et al., 2016, Interaction between basigin and monocarboxylate transporter 2 in the mouse testes and spermatozoa. Asian journal of andrology, 18(4): p. 600.

204. Kishimoto, A., et al., 2015, Immunohistochemical localization of GLUT3, MCT1, and MCT2 in the testes of mice and rats: the use of different energy sources in spermatogenesis. Biomedical Research, 36(4): p. 225-234.

205. Nakai, M., L. Chen, and R.A. Nowak, 2006, Tissue distribution of basigin and monocarboxylate transporter 1 in the adult male mouse: A study using the wild-type and basigin gene knockout mice. The Anatomical Record, 288(5): p. 527-535.

206. Lee, K.-H., et al., 2007, Expression of monocarboxylate transporters (MCTs) and basigin and estrogen receptor α (ERα)-mediated regulation of MCT1 expression in the efferent ductules of male reproductive tract during postnatal development. Journal of Animal Science and Technology, 49(3): p. 309-320.

207. Lee, K., 2008, Postnatal Ontogeny of Expression of Monocarboxylate Transporters (MCTs) and Two Regulatory Proteins, Basigin and Embigin, in The Epididymis of Male Rat. Journal of Animal Science and Technology, 50(1): p. 45-56.

208. Rato, L., et al., 2012, Metabolic modulation induced by oestradiol and DHT in immature rat Sertoli cells cultured in vitro. Bioscience Reports, 32(1): p. 61-69.

209. Boussouar, F., et al., 2003, Developmental and hormonal regulation of the monocarboxylate transporter 2 (MCT2) expression in the mouse germ cells. Biology of reproduction, 69(3): p. 1069-1078.

210. Oliveira, P., et al., 2012, Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta (BBA)-General Subjects, 1820(2): p. 84-89.

Aquaporins in Sertoli cells

Introduction

Aquaporins (AQPs) constitute a family of channel proteins permeating water, small solutes and certain gases across biological membranes [1]. Mammals have 13 homologues (AQP0-12) playing a number of roles. AQPs genes are found all kingdoms, which highlights their functional significance in living cells. The 2003 Nobel Prize for Chemistry was awarded to Peter Agre for the discovery and functional demonstration [2] of the AQP family of membrane channels (for a letter about Peter Agre, see [3]). Based on their biophysical properties of transport and phylogenesis, mammalian AQPs are grouped into orthodox AQPs, AQPs primarily permeable to water (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) and aquaglyceroporins, AQPs transporting a series of small uncharged solutes, particularly glycerol, in addition to water (AQP3, AQP7, AQP9, AQP10). AQP11 and AQP12, two AQPs whose conducting properties are unclear, are considered unorthodox homologues due to their distinct evolutionary pathway. Due to its marked ability to transport ammonia and hydrogen peroxide (besides to water), AQP8 is also indicated as ammoniaporin or peroxiporin.

The expression, biological significance and translational value of AQPs have been the object of intense investigation in all body districts. Unanticipated roles are being found for this family of channels besides more predictable functions. Here, we attempt an overview comprising the most recent advances and research trends on the regulation and function of AQPs in Sertoli cells (SCs), both in health and disease.

The Sertoli cell: a brief overview

The testes are functionally compartmentalized organs, being divided into seminiferous tubules and interstitial space, where spermatogenesis and testosterone biosynthesis take place, respectively. SCs are the somatic cellular component which are essential for testis formation and are responsible for the compartmentalization of this organ and the support of spermatogenesis [4]. Adjacent SCs are connected by tight junctions, creating a tight barrier known as Sertoli/blood-testis barrier (BTB), which allows them to create a protected environment within the seminiferous tubules [5]. These somatic cells, also called testicular “nurse cells”, play five essential roles that allow the occurrence of the spermatogenic event: (1) formation of the BTB; (2) nourishment and structural support to the developing germ cells; (3) elimination of defective germ cells; (4) production and release of fluid tubular seminiferous and other regulatory factors; (5) creation of an immune-privileged environment [4, 6]. SCs are cells of large dimensions, with columnar shape, which are adherent to the basal lamina. They

extend from the base of the seminiferous tubule to its lumen, where spermatozoa are released. These cells exhibit several particular features including large quantities of mitochondria, lipid droplets, glycogen particles and specific hormone receptors [7-10]. In fact, hormones are crucial regulatory factors for the functioning of the SCs [11, 12], particularly gonadotrophins (mainly follicle stimulating hormone - FSH), sex steroid hormones, thyroid hormones and insulin. For instance, compelling evidence has shown that FSH is essential for the establishment of male reproductive potential and particularly for SC physiology. SCs also possess a large variety of membrane transport proteins in their membranes, allowing them to control the seminiferous fluid composition and pH [13-16].

Testicular metabolic cooperation between Sertoli:germ cells: a selective process of nutrients and fluids

The metabolism of SCs is a central feature for the normal occurrence of spermatogenesis and presents some unique characteristics. As referred, SCs regulate the selective passage of substances from the interstitial fluid to the adluminal compartment that is filled with the seminiferous tubular fluid (STF) [17]. Among these substances, we must emphasize the importance of energy metabolites. It is known that SCs produce large amounts of lactate, mostly by the conversion of glucose via glycolysis [18]. Notably, the SC-produced lactate is then used by germ cells under development that are incapable of using glucose as an energetic source. In fact, lactate is the ideal metabolic substrate for developing germ cells [19] and acts as an anti-apoptotic factor in these cells [20], through mechanisms that remain a matter of debate. In sum, SCs present a Warburg-like glucose metabolism, similar to what is observed in cancer cells [21], favoring the fermentative (rather than oxidative) metabolism of glucose, despite being a less effective pathway in terms of ATP production [22].

In SCs, glucose crosses the plasma membrane through specific glucose transporters (GLUTs). Up until now, four GLUTs isoforms have been consistently reported in the plasma membrane of SCs, namely GLUT1, GLUT2, GLUT3 and GLUT4 [23-26]. After glucose crosses the plasma membrane, it is converted into pyruvate via glycolysis. Most of the pyruvate originated from glycolysis is converted by lactate dehydrogenase (LDH) into lactate, which is the preferred metabolic substrate of developing germ cells [27, 28]. The lactate produced is then released to the STF by specific membrane transporters present on SCs. So far, only monocarboxylate transporters (MCTs), particularly MCT1 and MCT4, have been implicated in the export of lactate by these cells [29]. The MCTs are responsible for the export of lactate to the intratubular fluid, where it may be used by developing germ cells [27, 30, 31] (Figure 1.6). Besides being the main energy source for ATP production by developing germ cells [11, 28], lactate is also

thought to be essential for the control of the STF pH, since lactate transport is coupled with H^+ and thus, a shifting of pH can be expected.

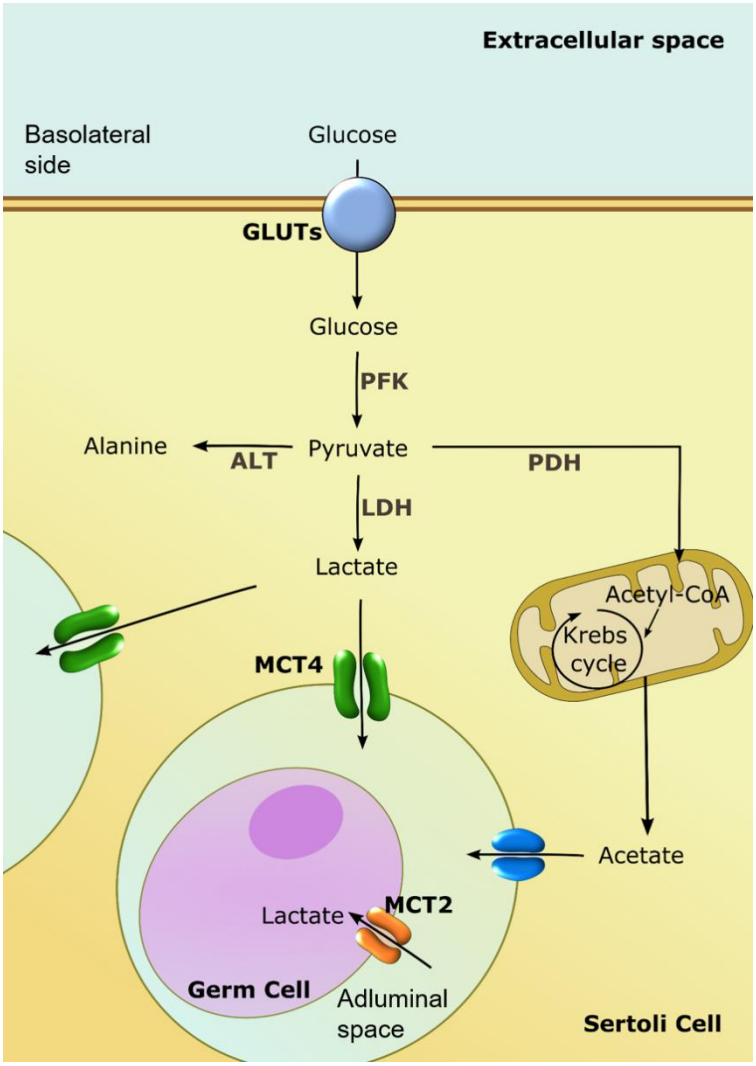


Figure 1.6 Schematic diagram of the metabolic cooperation between Sertoli cells (SCs) and developing germ cells. The glucose taken up from the extracellular space enters SCs through glucose transporters (GLUTs). The glucose is then converted to pyruvate through glycolysis. Pyruvate can follow multiple pathways. However, in these cells, most glucose is used to produce, via lactate by lactate dehydrogenase (LDH). Lactate is then transported out of SCs by specific monocarboxylate transporters (MCT4). Germ cells take up the lactate produced by SCs through MCT2. Of note, as happens in other cells, pyruvate can also be converted into alanine (by alanine aminotransferase - ALT) or transported to mitochondria forming acetyl-CoA (by pyruvate dehydrogenase - PDH). Acetyl-CoA is then converted into acetate that may be used by germ cells for lipid synthesis. PFK, phosphofrutokinase.

Recently, it has been described that, in addition to lactate, SCs also release high amounts of acetate to the extracellular environment [32]. However, the exit route from SCs or the role of acetate in spermatogenesis is not yet fully known. The authors suggested that it may be useful to maintain the elevated rate of lipid synthesis that is necessary for the germ cells division, being an intermediate for the synthesis of cholesterol and fatty acids [33] and thus, pivotal for the formation of membranes (Figure 1.6). Hence, spermatogenesis is completely dependent on the metabolic cooperation between SCs and developing germ cells [9]. In fact, compelling evidence suggests that alterations in these metabolic processes may result in deleterious outcomes for the reproductive potential of males or be involved in subfertility, or even infertility, induced by several diseases [34]. Cells from the germ line are completely dependent on carbohydrate metabolism (both on the aerobic and anaerobic pathways) [35]. On the other hand, sperm cells, which lie in the adluminal compartment, exhibit a great metabolic flexibility, using different metabolic pathways for the energy production [19] while spermatocytes exclusively depend on lactate supply by SCs [29, 35]. Lactate and pyruvate are known to be essential to germ cells at later developmental stages for energy production [36, 37]. The fact that the testes are oxygen-deprived organs [38] can explain why germ cells may use these distinctive metabolic pathways to obtain energy in their different stages of development. Indeed, several studies have demonstrated that spermatogenesis is completely dependent on the metabolic cooperation established between testicular cells and on the production of lactate by SCs. This cooperation is also known to be controlled by fluids composition and pH. In fact, and although this is a matter that has been so far overlooked, some very fine reports have shown the relevance of fluids for this process in the last few years.

Expression and subcellular localization of aquaporins in Sertoli cells

Fluid absorption and secretion are vital processes that occur in the male reproductive tract [39, 40]. Water movements are essential in determining the composition of the luminal fluids that fill the testicular ducts and for providing a means of transport to the spermatozoa into the epididymal ducts. Therefore, it is not surprising that AQPs have emerged as pivotal players in those mechanisms and that the expression of various AQPs has been described in the several testicular cells [41-45], which is consistent with the occurrence of water-dependent fluid movement in testes.

As said, SCs are the main mechanism responsible for the secretion of the fluid that fills the seminiferous tubules [46] and this mechanism are expected to rely on the participation of the various AQPs isoforms already described in these cells. While the presence of the majority of the known AQP isoforms has been reported to be present in the testis and/or in the ducts of the male reproductive tract [47-49], only a few of those isoforms (AQP0, AQP4, AQP8 and

AQP9) are known to be expressed in the SCs, although the data available is not always consistent and very few studies addressed the functional aspects of these channels (Table 1.1) [48, 50].

Table 1.1 Sertoli cell distribution and possible functions of mammalian Aquaporins (AQPs).

Aquaporin	Testicular distribution	Suggested function
AQP0	Sertoli cells and Leydig cells	Establishment of an adequate luminal environment in the seminiferous tubule; Transport of water from interstitial space into the lumen of the seminiferous tubule, in order to promote the movement of spermatozoa into the epididymal ducts
AQP4	Sertoli cells	Regulation of extracellular space volume, potassium buffering, fluid circulation and reabsorption
AQP8	Sertoli cells and germ cells	Formation of the seminiferous tubular fluid
AQP9	Sertoli cells, Leydig cells, spermatocytes, efferent ducts, epididymis	Transport of water and non-charged solutes in Leydig cells Formation of the seminiferous luminal fluid

AQP0 expression in the seminiferous epithelium seems to be restricted to SCs. In these cells, AQP0 is expressed in a specific semicircular pattern, which changes in the different stages of spermatogenesis [44]. Because AQP0 is expressed in SCs at stages VI–VIII of the spermatogenic cycle, it has been suggested that this AQP is involved in the transport of water from the interstitial space into the lumen of the seminiferous tubules during those specific periods, which correlate with the release of the elongating spermatids into the lumen of the seminiferous tubule. Thus, AQP0 seems to promote the movement of spermatozoa into the epididymis by facilitating the transport of water into the lumen of the seminiferous tubules. Nevertheless, further studies are needed to unveil the functional relevance of AQP0 for the movement of spermatozoa into the epididymis, since it is a pivotal event for the reproductive success of the males.

Regarding AQP4, limited data is available concerning its presence in the male reproductive tract. Recent data described its presence in rat SCs [51]. Actually, AQP4 is one of the more abundantly expressed AQPs in the equine testis [49]. Yet, no data is available on the function of this AQP in the various testicular cells or specifically in SCs. Nonetheless, as AQP4 is abundantly expressed in cells that support the blood-brain barrier [52], a structure similar to the BTB [53], playing a central role in water balance and ion homeostasis in the brain, it has been suggested to serve an analogous function in the testes and particularly in SCs (Table 1.1).

AQP8 was also identified in rat SCs [43, 54]. Interestingly, in the rat seminiferous epithelium, the expression of AQP8 was exclusively localized on SCs [43], where it is found homogeneously in every tubule, which is consistent with a constitutive expression of this AQP in SCs [55]. However, in contrast to the abundance of AQP8 in rat testis, AQP8 seems to be absent in the human testis [56]. Based on the role that this AQP plays in the cells where it is expressed, the presence of AQP8 in SCs suggests that it may be involved in the transport of water from the interstitial space into the lumen of the tubules. This movement of water seems to occur along an osmotic gradient, originated by the action of several other membrane transporters [57] (particularly the Na^+/K^+-pump, which has been co-localized with AQP8 on the adluminal portion plasma membranes of SCs) [43]. The wide presence of this AQP in the adluminal plasma membrane of SCs at all stages of the cycle of the seminiferous epithelium, leads to the suggestion that it may cooperate with other AQP isoforms in the seminiferous epithelium [43, 54]. It has been proposed that the transition of water into the lumen of the seminiferous tubules may be enhanced by the cooperation of other AQP isoforms which co-localize with AQP8 in SCs (such as AQP0). However, somewhat unexpectedly, Aqp8 null mice exhibited only mild phenotype differences on the reproductive organs when compared with the wild-type and $Aqp8^{-/+}$ heterozygous mice. Moreover, even though testis weight and size in $Aqp8^{-/-}$ mice were increased, no significant alteration on sperm parameters or impaired fertility were described in these rats [58].

Lastly, the presence of AQP9 has been detected at high levels throughout the male reproductive tract, even though AQP9-null mice are fertile [59]. The data available suggests that the expression of AQP9 is cell-specific in testes [43], with its presence being reported in rodent germ cells (particularly spermatocytes at early developmental stages) [45] and in the plasma and intracellular membranes of interstitial Leydig cells [43, 60]. AQP9 expression has also been reported in SCs [45, 61], suggesting that this AQP may play an essential role in the transport of water and/or non-charged solutes in all these testicular cells [62], similar to what happens in astrocytes, the key components of the blood-brain barrier.

Aquaporins functionality in testis and their possible relevance for Sertoli cell metabolism

While it has been described that orthodox AQPs, such as AQP0 and AQP4, are mostly permeable to water, being responsible for the establishment of cellular and/or transcellular fluxes, other non-conventional AQP isoforms are known to be permeable to additional non-charged solutes and play distinct roles in the physiology of the cells and tissues where they are expressed [63]. In the testicular tissue, and particularly in the seminiferous tubules, it has been suggested that both AQP0 and AQP4 should participate in water balance and ion

homeostasis, and that the presence of these transporters in SCs may be part of a mechanism that helps to create a route that facilitates the transepithelial movement of water in the seminiferous tubules [44, 51]. As previously mentioned, these water movements are essential for providing a means of transport to spermatozoa release into the lumen of the tubules into the excurrent duct system and also for controlling the composition of the luminal fluid that fills the seminiferous tubules (Figure 1.7).

Regarding AQP8, it has been proposed that this AQP could be involved in the transcellular movement of water from the interstitial space into the lumen of the seminiferous tubules [50]. The finding that AQP0 is expressed at high levels in these cells during specific stages of the spermatogenic cycle suggests that it may assist AQP8 in its function. As previously mentioned, AQP8 was described as being present in the adluminal plasma membrane of SCs [43] and should be responsible from the efflux of water from the SCs, while AQP0 might be involved in its uptake from the interstitial fluid (Figure 1.7). Therefore, the presence of these two AQPs at the specific time point of the cycle may facilitate the transport of water into the lumen and hence the movement of the spermatozoa out of the seminiferous tubules.

Contrastingly, AQP9 is an aquaglyceroporin that might be involved in distinct events in the testicular tissue other than the movement of water. As referred, AQP9 is permeable to water, urea, glycerol and monocarboxylic acids, namely lactic acid and acetic acid, being impermeable to cyclic sugars (e.g. D-glucose) [64-66]. The expression of AQP9 in the seminiferous epithelium [45, 61] and its selective permeability lead to the suggestion that this AQP might be involved in the transport of non-charged energy metabolites in the BTB. Similarly, to what happens in the blood-brain barrier (and particularly in astrocytes) [64], the presence of AQP9 in SCs supports a role in testicular metabolism as a glycerol and monocarboxylic acid channel. As discussed before, although glucose is a major source of energy for most testicular cells, developing germ cells that lie beyond the BTB depend on lactate as an energetic substrate [34]. The presence of AQP9 in SCs suggests that it may facilitate the diffusion of lactate to the intratubular fluid in conjunction with the MCTs already identified in these cells, particularly MCT4, which is reported to be highly expressed in SCs [9]. We may also hypothesize that glycerol can diffuse through AQP9 channels into SCs, although the current knowledge concerning the metabolism of this polyol in the testicular cells is scarce. Still, it has been described that an increase in the testicular levels of glycerol (by exogenous administration) transiently compromises spermatogenesis, also leading to disruption of the BTB [67]. Taking this into account, AQP9 may play a crucial role in the success of spermatogenesis, particularly in pathological conditions associated with increased plasma glycerol levels (e.g. obesity, diabetes mellitus). Taken together, the data available raises the hypothesis that AQP9 in SCs may play a role in testicular energy metabolism and metabolic

cooperation as a lactate (glycerol) channel. This hypothesis still waits functional validation and the molecular mechanisms by which AQP9 may control the metabolic cooperation between SC:germ cells are still unknown.

Therefore, there is much to unveil concerning the function of AQPs in the mammalian cells of the male reproductive tract, particularly in SCs, and their role in the processes that define a successful functional sperm production. However, there are recent compelling evidences suggesting that AQPs are crucial for normal spermatogenesis and, thus, for overall male reproductive health. Nevertheless, their role and relevance for male fertility remains a matter of intense discussion. It is expected that dysregulation of AQPs function may be involved in the subfertility, or even infertility, induced by several diseases in males. This is a research scope that has been so far overlooked. The role that AQPs play in male fertility deserves a special attention from researchers in the years to come, and may represent an exciting field, to unveiling novel mechanisms to control male fertility.

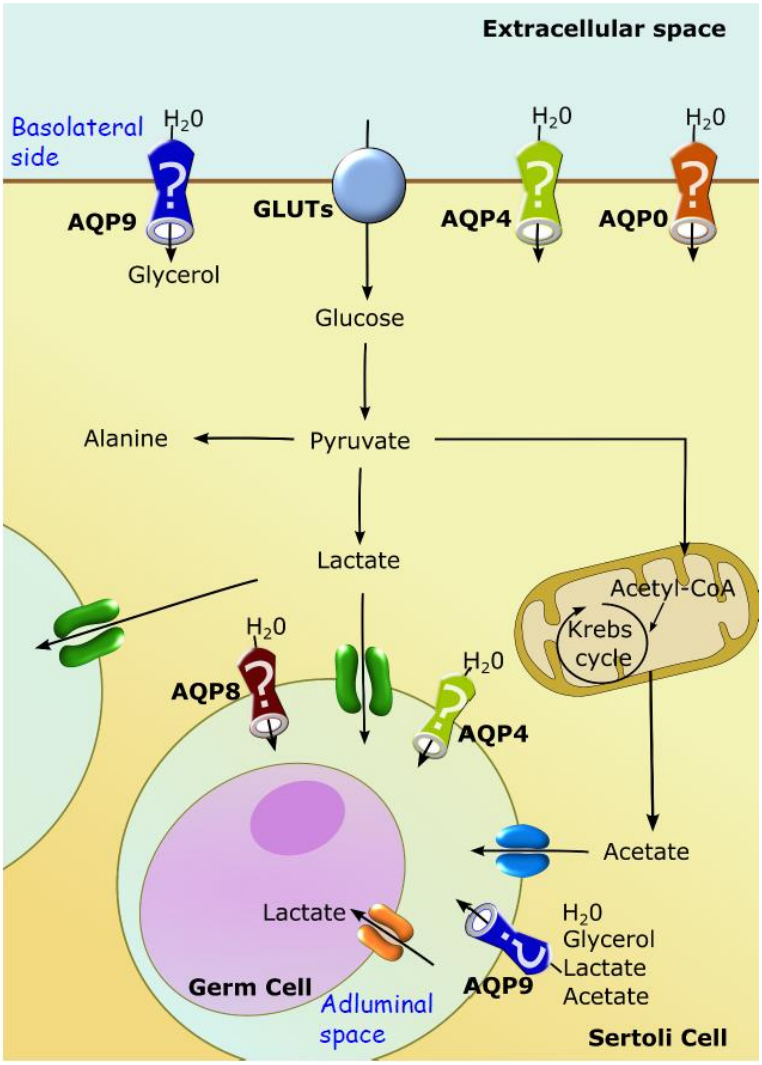


Figure 1.7 Schematic diagram of the possible subcellular localizations of aquaporins (AQPs) in Sertoli cells (SCs) and their possible link to metabolism. SCs express multiple AQP homologues, AQP0, AQP4, AQP8 and AQP9. Basolateral plasma membrane is believed to contain AQP0, AQP4 and AQP9. Basolateral AQP9 may be relevant for testicular metabolic cooperation. The SC adluminal plasma membrane contains AQP4, AQP8 and AQP9. Adluminal AQP9 may mediate the extrusion of metabolic intermediates such as lactate, acetate and glycerol (in addition to water). The functional significance of adluminal AQP4, an AQP highly permeable to water, and AQP8, a homologue conducting water and some other molecules, remains elusive.

References

1. Agre, P., 2004, Aquaporin water channels. Bioscience reports, 24(3): p. 127-163.
2. Preston, G.M., et al., 1992, Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science, 256(5055): p. 385-387.
3. Calamita, G. and P. Portincasa, 2016, The power of science diplomacy, a lesson from the Nobel laureate Peter Agre. European journal of clinical investigation, 46(5): p. 491-493.

4. Oliveira, P.F. and M.G. Alves, The Sertoli Cell at a Glance, in Sertoli Cell Metabolism and Spermatogenesis, M. Owen, Editor. 2015, Springer. p. 3-13.
5. Hadley, M.A., et al., 1985, Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. Journal of cell biology, 101(4): p. 1511-1522.
6. Mruk, D.D. and C.Y. Cheng, 2004, Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocrine reviews, 25(5): p. 747-806.
7. Martins, A.D., et al., 2014, Physiology of na+/h+ exchangers in the male reproductive tract: relevance for male fertility. Biology of Reproduction, 91(1): p. 11, 1-6.
8. Bernardino, R.L., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current medicinal chemistry, 20(32): p. 4037-4049.
9. Rato, L., et al., 2012, Metabolic regulation is important for spermatogenesis. Nature Reviews Urology, 9(6): p. 330-338.
10. Guo, T.B., et al., 2003, Evidence for a Role of Glycogen Synthase Kinase-3β in Rodent Spermatogenesis. Journal of andrology, 24(3): p. 332-342.
11. Rato, L., et al., 2015, New insights on hormones and factors that modulate Sertoli cell metabolism. Histology and histopathology, 31(5): p. 499-513.
12. Bernardino, R.L., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current Medicinal Chemistry, 20(32): p. 4037-49.
13. Oliveira, P., et al., 2009, Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. Journal of Membrane Biology, 227(1): p. 49.
14. Oliveira, P.F., et al., 2009, Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction, 137(2): p. 353-359.
15. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. The Journal of membrane biology, 236(2): p. 215-224.
16. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7):p. 179-188.
17. Setchell, B., 1980, The functional significance of the blood-testis barrier. Journal of andrology, 1(1): p. 3-10.
18. Robinson, R. and I.B. Fritz, 1981, Metabolism of glucose by Sertoli cells in culture. Biology of reproduction, 24(5): p. 1032-1041.
19. Boussouar, F. and M. Benahmed, 2004, Lactate and energy metabolism in male germ cells. Trends in endocrinology and metabolism, 15(7): p. 345-350.

20. Erkkilä, K., et al., 2002, Lactate inhibits germ cell apoptosis in the human testis. Molecular human reproduction, 8(2): p. 109-117.
21. Oliveira, P.F., et al., 2015, The Warburg effect revisited—lesson from the Sertoli cell. Medicinal research reviews, 35(1): p. 126-151.
22. Grootegoed, J., et al., 1986, Metabolism of radiolabelled energy-yielding substrates by rat Sertoli cells. Journal of reproduction and fertility, 77(1): p. 109-118.
23. Carosa, E., et al., 2005, Ontogenetic profile and thyroid hormone regulation of type-1 and type-8 glucose transporters in rat Sertoli cells. International journal of andrology, 28(2): p. 99-106.
24. Galardo, M.N., et al., 2008, Regulation of expression of Sertoli cell glucose transporters 1 and 3 by FSH, IL1β, and bFGF at two different time-points in pubertal development. Cell and tissue research, 334(2): p. 295-304.
25. Kokk, K., et al., 2003, Immunohistochemical detection of glucose transporters class I subfamily in the mouse, rat and human testis. Medicina (Kaunas, Lithuania), 40(2): p. 156-160.
26. Ulisse, S., et al., 1992, Thyroid hormone stimulates glucose transport and GLUT1 mRNA in rat Sertoli cells. Molecular and cellular endocrinology, 87(1): p. 131-137.
27. Oliveira, P.F., et al., 2012, Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et biophysica acta, 1820(2): p. 84-89.
28. Oliveira, P.F. and M.G. Alves, Sertoli Cell Metabolism and Spermatogenesis. 2015, United Kingdom: Springer.
29. Galardo, M.N., et al., 2007, The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-bD-ribonucleoside, regulates lactate production in rat Sertoli cells. Journal of molecular endocrinology, 39(4): p. 279-288.
30. Oliveira, P.F., et al., 2011, Influence of 5α-dihydrotestosterone and 17β-estradiol on human Sertoli cells metabolism. International journal of andrology, 34(6pt2): p. e612-e620.
31. Mannowetz, N., P. Wandernoth, and G. Wennemuth, 2012, Basigin interacts with both MCT1 and MCT2 in murine spermatozoa. Journal of cellular physiology, 227(5): p. 2154-2162.
32. Alves, M.G., et al., 2012, In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1823(8): p. 1389-1394.
33. Yoshimoto, M., et al., 2001, Characterization of acetate metabolism in tumor cells in relation to cell proliferation: acetate metabolism in tumor cells. Nuclear medicine and biology, 28(2): p. 117-122.

34. Alves, M.G., et al., 2013, Molecular mechanisms beyond glucose transport in diabetes-related male infertility. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1832(5): p. 626-635.
35. Bajpai, M., G. Gupta, and B. Setty, 1998, Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. European journal of endocrinology, 138(3): p. 322-327.
36. Jutte, N.H., et al., 1981, Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. Journal of Reproduction and Fertility, 62(2): p. 399-405.
37. Nakamura, M., H. Akiya, and K. Junzo, 1981, Stimulation of protein synthesis in round spermatids from rat testes by lactate. II. Role of adenosine triphosphate (ATP). Journal of biochemistry, 90(4): p. 933-940.
38. Wenger, R.H. and D.M. Katschinski, 2005, The hypoxic testis and post-meiotic expression of PAS domain proteins. in Seminars in cell & developmental biology. Seminars in Cell and Developmental Biology, 16(4-5): p. 547-553.
39. Levine, N. and D.J. Marsh, 1971, Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. The Journal of Physiology, 213(3): p. 557-570.
40. Russell, L.D., A. Bartke, and J.C. Goh, 1989, Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion and flow. The American journal of anatomy, 184(3): p. 179-89.
41. Huang, H.-F., et al., 2006, Function of aquaporins in female and male reproductive systems. Human Reproduction Update, 12(6): p. 785-795.
42. Sohara, E., S. Uchida, and S. Sasaki, Function of Aquaporin-7 in the Kidney and the Male Reproductive System, in Aquaporins, B. E, Editor. 2004, Springer: Germany. p. 219-231.
43. Badran, H.H. and L.S. Hermo, 2002, Expression and Regulation of Aquaporins 1, 8, and 9 in the Testis, Efferent Ducts, and Epididymis of Adult Rats and During Postnatal Development. Journal of Andrology, 23(3): p. 358-373.
44. Hermo, L., D. Krzeczunowicz, and R. Ruz, 2004, Cell Specificity of Aquaporins 0, 3, and 10 Expressed in the Testis, Efferent Ducts, and Epididymis of Adult Rats. Journal of Andrology, 25(4): p. 494-505.
45. Yeung, C.H., et al., 2010, Aquaporins in the human testis and spermatozoa - identification, involvement in sperm volume regulation and clinical relevance. International Journal of Andrology, 33(4): p. 629-41.
46. Sprando, R.L. and L.D. Russell, 1987, Comparative study of cytoplasmic elimination in spermatids of selected mammalian species. American Journal of Anatomy, 178(1): p. 72-80.

47. Hermo, L. and C.E. Smith, 2011, Thirsty business: cell, region, and membrane specificity of aquaporins in the testis, efferent ducts, and epididymis and factors regulating their expression. Journal of andrology, 32(6): p. 565-575.
48. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. Journal of Membrane Biology, 236(2): p. 215-24.
49. Klein, C., M. Troedsson, and J. Rutllant, 2013, Region-Specific Expression of Aquaporin Subtypes in Equine Testis, Epididymis, and Ductus Deferens. Anatomical Record, 296(7): p. 1115-1126.
50. Jesus, T.T., et al., 2014, Aquaporin-4 as a molecular partner of cystic fibrosis transmembrane conductance regulator in rat Sertoli cells. Biochemical and biophysical research communications, 446(4): p. 1017-1021.
51. Iacovetta, C., E. Rudloff, and R. Kirby, 2012, The role of aquaporin 4 in the brain. Veterinary clinical pathology, 41(1): p. 32-44.
52. Alves, M.G., et al., 2012, Impact of diabetes in blood-testis and blood-brain barriers: resemblances and differences. Current diabetes reviews, 8(6): p. 401-412.
53. Tani, T., et al., 2001, Immunolocalization of aquaporin-8 in rat digestive organs and testis. Archives of histology and cytology, 64(2): p. 159-68.
54. Elkjar, M.-L., et al., 2001, Immunolocalization of aquaporin-8 in rat kidney, gastrointestinal tract, testis, and airways. American journal of physiology. Renal physiology, 281(6): p. F1047-1057.
55. Koyama, N., et al., 1998, Cloning and functional expression of human aquaporin8 cDNA and analysis of its gene. Genomics, 54(1): p. 169-72.
56. Gravis, C., R. Yates, and I. Chen, 1976, Light and electron microscopic localization of ATPase in normal and degenerating testes of Syrian hamsters. American Journal of Anatomy, 147(4): p. 419-431.
57. Yang, B., et al., 2005, Phenotype analysis of aquaporin-8 null mice. American journal of physiology. Cell physiology, 288(5): p. C1161-1170.
58. Rojek, A.M., et al., 2007, Defective glycerol metabolism in aquaporin 9 (AQP9) knockout mice. Proceedings of the National Academy of Sciences, 104(9): p. 3609-3614.
59. Elkjaer, M., et al., 2000, Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. Biochemical and biophysical research communications, 276(3): p. 1118-28.
60. Jesus, T.T., et al., 2014, Aquaporin-9 is expressed in rat Sertoli cells and interacts with the cystic fibrosis transmembrane conductance regulator. International Union of Biochemistry and Molecular Biology Life, 66(9): p. 639-44.
61. Nihei, K., et al., 2001, Immunolocalization of aquaporin-9 in rat hepatocytes and Leydig cells. Archives of histology and cytology, 64(1): p. 81-88.

62. Cho, Y., M. Svelto, and G. Calamita, 2003, Possible functional implications of aquaporin water channels in reproductive physiology and medically assisted procreation. Cellular and molecular biology 49(4): p. 515-519.
63. Alves, M.G., et al., 2015, CFTR regulation of Aquaporin-mediated water transport: a target in male fertility. Current drug targets, 16(9): p. 993-1006.
64. Badaut, J. and L. Regli, 2004, Distribution and possible roles of aquaporin 9 in the brain. Neuroscience, 129(4): p. 969-979.
65. Holm, L.M., et al., 2005, NH3 and NH 4+ permeability in aquaporin-expressing Xenopus oocytes. Pflügers Archiv : European journal of physiology, 450(6): p. 415-428.
66. Carbrey, J.M., et al., 2003, Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. Proceedings of the National Academy of Sciences, 100(5): p. 2945-2950.
67. Wiebe, J.P., et al., 2000, Glycerol disrupts tight junction-associated actin microfilaments, occludin, and microtubules in Sertoli cells. Journal of andrology, 21(5): p. 625-635.

Formation and biochemistry of seminal plasma and male accessory fluids

Abstract

An appropriate microenvironment in each segment of the male reproductive tract is crucial for the successful maturation and motility of sperm and thereby for male fertility. Spermatozoa are produced in the testes and transported to the epididymis along with the seminiferous tubular fluid. The epididymis has an epididymal milieu that maintains the optimal conditions needed for sperm maturation and storage. The composition of the epididymal luminal fluid is gradually changed throughout the epididymal duct due to absorption and secretion processes. The main changes in epididymal fluid and other fluids produced by accessory glands is the ionic content, osmolality, pH and spermatocrit. Sperm motility is a good predictor of human male fertility that is controlled by some parameters, such as bicarbonate and calcium concentrations, that constantly fluctuate throughout the reproductive ducts. The spermatozoa leaving the epididymis along with the epididymal fluid, will join to secretions from the prostate and seminal vesicles, thus constituting the seminal plasma. More attention should be paid to the fluids surrounding the sperm, namely its ionic composition and pH in order to unravel the causes of idiopathic infertility, which represents an elevated percentage of infertile men.

Keywords: Seminiferous tubular fluid, Spermatozoa, Epididymis, Epididymal fluid, Seminal vesicles, Seminal plasma, prostate, ions, ionic transporters, pH, bicarbonate, calcium, sperm capacitation, motility.

Introduction

The male reproductive system is complex and highly sophisticated. Spermatozoa are generated in the testes and undergo a maturational process while traveling through the long epididymal tubule, until reach the cauda epididymis. The sperm are continuously exposed to a specialized luminal fluid microenvironment. The composition of the fluid that bathes spermatozoa is unique in all ducts of the male reproductive tract and is different from blood plasma and interstitial fluid [1]. The composition of the testicular fluid and epididymal luminal fluid is variable, and major changes from one region to the next were detected. The formation of seminal fluid is essential for male reproduction, since the biochemical composition of this fluid is critical for sperm function. Seminal fluid contributes to the process of sperm capacitation and fertilization, controlling pH, nourishing spermatozoa and creates a proper environment for

immunological protection [2]. The motility of spermatozoa is also severely affected by the composition of seminal plasma. In fact, it is facilitated by a controlled pH of seminal fluid [3]. Failure to maintain pH homeostasis along the male reproductive tract may impair the production and maturation of spermatozoa and therefore causing subfertility or infertility [4, 5]. The importance of understanding functional relevance of the luminal fluids of the male reproductive tract is related to the fact that up to 40% of infertile men present idiopathic infertility, which may be a reflection of disorders in sperm maturation and storage [6]. This chapter will discuss some of the key components of the testicular fluid, epididymal fluid and seminal plasma and briefly understand how this microenvironment is created.

The epididymal milieu

The epididymis is an organ consisting of a highly convoluted duct system supposed to cover a total length of about 20 meters in man. All this duct is strictly dependent upon testicular products for the maintenance of its structure as well as secretory, reabsorptive, biosynthetic and metabolic activity [7, 8]. The epididymal lumen has usually the most complex fluid found in any exocrine gland. This may be due to the continuous changes in composition, such as the presence of substances in unusually high concentrations for unknown reasons, or those not found in other body fluids [9]. The epididymal fluid is largely influenced by the end-product of the exocrine activity of the testes, testicular fluid or testicular plasma. Besides being dependent on the presence of androgens (e.g.: testosterone) [10], the epididymis also relies on the presence of luminal fluid factors obtained from the testes and the epididymis itself. Some studies described that without testicular luminal fluid factors many cells within initial segment of epididymis undergo apoptosis [11, 12]. Therefore, factors originated from testes are responsible for the preservation of the integrity and survival of cells within epididymis. The testicular fluid that goes to epididymis is mainly composed by the seminiferous tubular fluid (STF). There is a layer of spindle-shaped cells that fill the space among the external and internal lamella of the seminiferous tubules surrounding tissue, to which the tubules owe their contractibility [13]. STF was thought to be a mixture of primary fluid, most likely secreted by Sertoli cells [7]. However, the seminiferous epithelium secretes a fluid that is later modified when enters the rete testis, by alterations in potassium, bicarbonate, sodium, chloride, proteins and steroids [14]. For instance, the STF is characterized by being potassium-rich, which is later altered to a sodium chloride-rich, when entering the rete testis [7]. Thereby, the epithelium of rete testis is active in the regulation of the luminal fluid microenvironment, and not so in controlling the amount of fluid produced in the STF that goes to the epididymis [7]. Nevertheless, the role of the rete testis in the regulation of luminal fluid environment has been overlooked. The testicular fluid undergoes a sequence of changes before it entries into

epididymis, all long its passage through the seminiferous tubules, rete testis and lastly the efferent ducts. Therefore, the testicular fluid is composed of spermatozoa released by the germinal epithelium into a combination of fluids originated from different compartments of the testes [15]. Epididymal lumen has a complex fluid controlled by blood-epididymal barrier (BEB), which exists to maintain a specialized luminal fluid to provide a suitable environment for sperm maturation and survival [16]. Luminal fluid microenvironment has a different composition than that of blood plasma and interstitial fluid, thus supporting the hypothesis that epididymal luminal fluid is important in the processes of sperm maturation and storage. The epididymal epithelium is constituted by very active cells in intermediary metabolism that generate products of energy metabolism and reactive oxygen species [6, 16]. The epididymal lumen is rich in inorganic ions and small organic molecules which create an environment that is hyperosmotic relative to serum [17]. Perturbations in the microenvironment that surround the spermatozoa such as alterations in the luminal pH or extreme temperatures can affect maturation and development [4].

Luminal fluid composition: relevance of bicarbonate

The luminal fluid surrounding the spermatozoa is distinct between each region of male reproductive tract and is different from that of blood plasma. The process begins inside the testes where spermatozoa are released into the lumen of the seminiferous tubule. Inside of seminiferous tubule the spermatozoa are bathed by STF that is produced and released mainly by Sertoli cells [18]. The formation of the ionic composition of the STF includes the net movement of water, K^+ secretion and Cl^-, Na^+ and HCO_3^- reabsorption that contribute for luminal acidification [18]. Substantial differences in the ionic composition of STF have been reported comparatively to blood plasma, particularly concerning the concentration of K^+, Na^+, Cl^- and HCO_3^-. The reported composition of the STF differs according to the method of collection [19-22]. The study of male reproductive tract fluids is very complex, being micropuncture the most common method, which has originally been used to study renal physiology. The major problem of the micropuncture analysis is the quantity of sample since it obtains very low volumes in the order of nanoliters and picoliters [23]. Therefore, the analysis of these samples is really difficult, hence there are few studies focused on the composition of the fluids in human reproductive tract using this technique. Even though, STF collected from rat seminiferous tubules by micropuncture evidenced more K^+ than in fluid collected from rete testis, in a concentration about ten times higher compared to blood plasma. However, the HCO_3^- and Na^+ concentrations were lower in rat STF than blood plasma (Table 1.2). Ca^{2+} concentration in STF was similar to that of blood plasma and Mg^{2+} and phosphate were higher in STF. With a pH of approximately 7.3, STF is more acid that blood and the osmolality of the

fluid available inside the seminiferous tubules is isosmotic in the rat [1, 22-24] (Table 1.2). The electrical potential difference across the seminiferous tubules epithelium is approximately 4.8 mV, lumen negative, to the interstitial medium. The K^+ and Cl^- enter the tubule against their electrochemical gradient, whereas Na^+ and HCO_3^- entrance into the lumen is favored by the electrochemical gradient [24]. The STF flows through the rete testis and the efferent ducts into the epididymis. Rete testis fluid arises within the seminiferous tubules, thus containing some of the same components present in STF. Rete testis fluid contain more Na^+ and Ca^{2+} than STF, however the concentrations of K^+, phosphate and Mg^{2+} are lower [1, 22, 23] (Table 1.2).

Concentrations of ions, small organic solutes and proteins are dependent on the movement of water in and out of the lumen. Thus, the concentrations of ions in the epididymal luminal fluid are not only due to direct secretion into the lumen, but also to the volume of water that is reabsorbed. In many species, 70-96% of the fluid leaving the testes is reabsorbed in the efferent ducts. Particularly, in rats, 96% of the fluid is reabsorbed [14]. Studies in rats demonstrated that when the efferent duct connection is compromised for 24 hours there is a rise of about 50% in testicular volume due to accumulated fluid, thus illustrating the efficiency of the efferent ducts and epididymis to control the volume of fluid [25]. In addition, there is another parameter that demonstrates the absorption of water by the efferent ducts and initial segment of epididymis: the fractional volume occupied by the sperm (spermatocrit). The spermatocrit increases from 0.2 ± 0.02 in seminiferous tubules to 0.4 ± 0.2 in epididymal caput and to 0.5 ± 0.03 in cauda epididymis [24], indicating that about 50% of the fluid entering the caput is absorbed by the efferent ducts. Due to water reabsorption by vas deferens, in this section of the male reproductive tract the spermatocrit of 0.7 ± 0.02, higher 0.2 than in the epididymal cauda [24] (Table 1.2). Apparently, most of the reabsorption process occurs before the cauda of the epididymis, because the ion concentrations are not significantly altered when compared with vas deferens.

Micropuncture studies have showed that the concentration of Na^+ decreases and K^+ increases in the epididymal fluid along the epididymis (Table 1.2). This suggests that both Na^+ and water are reabsorbed by the epididymal epithelium. Some in vitro and in vivo studies performed in rats indicate that fluid reabsorption is a passive process and secondary to active Na+ transport [26-28]. The rates of the ionic transport of Na^+, Cl^-, K^+ and water have been found to fluctuate with the region of the duct, but it became clear that epididymis absorbs Na^+, Cl^- and water and secrete K^+. Notably, the more distal parts of the rat epididymis absorb Na^+ and water and secret K^+ at a higher rate than more distal parts of the epididymal duct [28]. Therefore, Na^+ is actively reabsorbed by the epididymal epithelium, thereby creating an osmotic gradient for water reabsorption. The ionic transport that occurs along the epididymis is associated with a progressive increase in the potential difference across the epithelial wall. It is 5.6 ± 0.1mV,

lumen negative, in caput of epididymis and increases to 27 ± 0.6, lumen negative, in cauda and vas deferens. The process of Na^+ reabsorption occurs against an electrochemical potential difference. The rising K^+ concentration can be mainly due to the removal of water, but there is also a contribution from epithelial secretion into the lumen [24]. Potassium concentration is quite different from epididymis and vas deferens to the blood plasma, whereby it is away from electrochemical equilibrium, and must involve a more complex process than simple equilibration. Moreover, Cl^- is also reabsorbed against an electrochemical gradient, but the rate of Cl^- reabsorption is only a fraction of the Na^+ reabsorption rate in the epididymis [24].

Table 1.2 Electrolytes concentrations, osmolality, pH and spermatocrit in the seminiferous tubular fluid, rete testis fluid, epididymis fluids, vas deferens fluid and plasma of the rat. STF- Seminiferous tubular fluid.

	STF	**Rete testis fluid**	**Epididymis fluids**			***Vas deferens* fluid**	**Plasma**
			caput	**corpus**	**cauda**		
Na^+ (mM/L)	110±5 [24]	142±10 [1, 22, 23]	104±3 [23]	94±11 [23]	37±3 [23]	23±4 [24]	139±4 [23]
K^+ (mM/L)	40±1 [22, 23]	12±0.5 [22, 23]	21±2 [23]	37±2 [24]	50±1 [30]	52±2 [24]	4±1 [1]
HCO_3^- (mM/L)	20±0.4 [1, 24]	21±5 [1, 22]	3±0.3 [24]	------	7±0.3 [24]	7±0.3 [24]	30±0.1 [24]
Cl^- (mM/L)	143±12 [23]	136±5 [1, 22, 23]	24±2 [23]	39±5 [23]	27±2 [23, 24]	11±2 [24]	122±4 [23]
Phosphate (mM/L)	9±1 [23]	2±0.5 [23]	59±5 [23]	94±3 [23]	84±5 [23]	----------	2±0.1 [23]
Ca^{2+} (mM/L)	0.4±0.03 [1, 23]	0.8±0.1 [1, 23]	0.9±0.1 [23]	0.5±0.1 [23]	0.3±0.1 [23]	----------	0.5±0.1 [23]
Mg^{2+} (mM/L)	1.2±0.2 [1, 23]	0.4±0.1[1 , 23]	2±0.3 [23]	3±0.2 [23]	1±0.1 [23]	----------	0.4±0.02 [23]
Osmolality (mOsm/Kg)	338±7 [22, 24]	328±23 [22]	315±4 [24]	340±8 [24]	329±5 [24]	339±4 [24]	311±3 [22, 24]
pH	7.3 [24, 29]	----------	6.6 [24, 29]	----------	6.9 [24]	6.9 [24]	7.5 [24]
Spermatocrit	0.2±0.02 [24]	----------	0.4±0.02 [24]	0.6±0.04 [24]	0.5±0.03 [24]	0.7±0.02 [24]	----------

Levine and Kelly [29] describe the first measurement of pH made *in vivo* in the male reproductive tract of rat. They were able to precisely localize the primary site of acidification in the initial segment of epididymis. The authors observed that in the beginning of the initial segment of epididymis the pH is about 7.20 ± 0.06 and in the end of the initial segment is 6.79 ± 0.06, so the fluid becomes more acid reaching 6.6 in caput [29]. The pH value of the epididymal fluid very slightly increases again at the end of the epididymis (Table 1.2).

Luminal acidification depends on several processes, such as HCO_3^- reabsorption and proton secretion that occurs in different cell types and in different regions of the epididymis. The most important physiological buffer system in mammals that controls and maintains pH range is HCO_3^-. The HCO_3^- originated in the testes (20 mM) is partially reabsorbed between the seminiferous tubules and the caput of epididymis by the HCO_3^- transporters [31] and by the action of carbonic anhydrases [32]. Bicarbonate reabsorption is essentially reached by the main cells in the initial segment of the epididymis [4]. In the rat epididymis, HCO_3^- concentration is around 3-7 mM, with a slight increase in the cauda and vas deferens, related to HCO_3^- reabsorption in the initial segment [4, 33]. The HCO_3^- is essential in luminal fluid for spermatogenesis and spermatozoa maturation. The intratubular concentration of HCO_3^- in epididymis and epididymal spermatozoa is regulated by HCO_3^- transporters, namely HCO_3^- permeable proteins and carbonic anhydrase activity [31]. Bicarbonate has been shown to stimulate mammalian sperm motility and metabolism through activation of adenylate cyclase. Soluble adenylyl cyclase (sAC) is a HCO_3^- chemosensor for immature and mature spermatozoa. *In vivo* studies showed that HCO_3^- modulates the enzymatic activity of sAC by direct interaction with allosteric site of the enzyme to produce cAMP under pH independent conditions [34]. Bicarbonate-regulated sAC was initially purified from testes cytosol, which is an alternative source for cAMP. sAC is not only a soluble protein but has well-defined intracellular targets, such as mitochondria, centrioles, mitotic spindles, midbodies and nuclei [35]. Therefore, adenylyl cyclases are in close association to cAMP effectors, cAMP are regulated by adenylyl cyclases and adenylyl cyclases are regulated by HCO_3^- [35]. High concentrations of HCO_3^- should be linked with high cAMP production and the reverse also occurs. Modulation of key ion channels, activation of the sAC/cAMP signaling pathway and protein phosphorylation events are essential for maintaining spermatozoa in a quiescent state during their maturation and storage in the epididymis only. During ejaculation and passage to the female reproductive tract, the increase on pH and HCO_3^- concentration induces sperm capacitation [35, 36].

Bicarbonate transporters in epididymis

Acid-base transporters are classified according to their functionality, into two distinct groups: acid-extruders (Na^+-H^+ exchangers, Na^+-driven HCO_3^-/Cl^- transporters, Na^+/HCO_3^- cotransporters, and V-ATPases), used to increase intracellular pH when acidosis occurs, and acid-loaders (Na^+-independent HCO_3^-/Cl^- transporters and Na^+/ HCO_3^- co-transporters), used to decrease intracellular pH when alkalosis occurs [37]. The function as acid loader or as acid extruder is dependent also on the ionic gradient established through the membranes [38].

Bicarbonate transporters are largely expressed and involved in the regulation of pH, cell migration, cell volume, transepithelial acid/base transport and Cl^- secretion. Like in other physiological systems, in male reproductive tract, the luminal HCO_3^- combines a proton to form H_2CO_3, which is then converted into CO_2 and H_2O by catalysis of carbonic anhydrase [32]. Since this reaction may be reversible in epithelial cells, the CO_2 is hydrated under the action of carbonic anhydrase and then dissociates into HCO_3^- and proton [31]. The involvement of HCO_3^- in epididymal proton secretion is suggested by studies showing that the use of an inhibitor (acetazolamide) of carbonic anhydrase causes an increase in luminal pH in the cauda epididymis [39]. The HCO_3^- is extruded via HCO_3^- transporters and proton is recycled into the lumen via V-type H^+-ATPase [40, 41] or Na^+-H^+ exchanger [42]. In male reproductive tract, the HCO_3^- transporters are highly expressed, particularly in the epididymis. The main families of HCO_3^- transporters are identified in epididymis, Solute carrier 4 (SLC4) and Solute carrier 26 (SLC26) family [5]. Intracellular and extracellular HCO_3^- are transported in and out of the cell across epididymal membrane mostly by the HCO_3^- exchanger (Anion exchanger - AE), or the Na^+-HCO_3^- cotransporter. The “housekeeping AE”, the AE2 is widely distributed in male reproductive tract [5, 43]. AE2 is identified in the initial segment, intermediate zone and caput epididymis and in relative low abundance in the distal regions, including the cauda epididymis [44, 45]. AE2 plays a key role on intracellular pH regulation and its functioning is stimulated by exposure to alkaline media and inhibited by the presence of acidic media [5]. The presence of AE2 in the basolateral membrane of the epithelial cells in the proximal parts of the epididymis, is associated with the low luminal HCO_3^- concentration attained in this segment. Along with other HCO_3^- transporters, AE2 might contribute to net HCO_3^- reabsorption. The expression of this important HCO_3^- transporter is increased in some pathological cases, such as it was observed in prediabetic-induced rats [44]. The significant increase of abnormal sperm morphology in prediabetic rats [46] was suggested to be related to the alteration of the HCO_3^- transepithelial epididymal fluxes.

The Na^+-dependent members of the SLC4 family also contribute to the transport of HCO_3^- in the epididymis. Due to their transport stoichiometry, they are divided into two sub-groups: electrogenic (NBCe) and electroneutral (NBCn). The direction and stoichiometry of the

transport depends on the tissues in which they are expressed. In epididymis, the NBCe1 and NBCn1 uptake the ions (HCO_3^- and Na^+) into the cytosol [44]. The NDCBE (Na^+-driven Cl^-/HCO_3^- exchanger) is an intracellular pH regulator that transports extracellular HCO_3^- and Na^+ in exchange for intracellular Cl^- and H^+, playing an essential role in cellular alkalinisation. The expression of NDCBE has also been detected throughout all the male reproductive tract, including in the epididymis, being that the expression is also altered in some pathological states (e.g. prediabetes) [44]. Bicarbonate-permeable members of the SLC26 family is the second major subfamily of HCO_3^- transporters and some members are also expressed along the male reproductive tract. However, their function is not so well studied as compared with SLC4 family. In epididymis, four members of HCO_3^- transporters of SLC26 family were identified: SLC26A3, SLC26A4, SLC26A6 and SLC26A7 [31, 44]. The SLC26A3 transporter is highly expressed in the testes, but has a low expression in the epididymis. It was only identified in the luminal side of the apical mitochondria-rich cells (AMRC), located in the caput epididymis and only occasionally in the corpus epididymis [47]. The expression of SLC26A4, SLC26A6 and SLC26A7 [48, 49] has also been identified in the epididymis. Still, functional studies concerning the role of some of these in male reproductive tract tissues are very scare or inexistent.

In addition to the discussed HCO_3^- transporters, CFTR is also expressed in epididymis and contributes to the ionic composition of the luminal fluid. CFTR has an important role in transepithelial salt absorption, as well as in the HCO_3^- secretion and regulation of fluid volume in epithelial cells [31]. The epithelial cells of the head epididymis present more intense expression of CFTR, whereas the distribution is irregular in the epithelial cells of the body and tail [50]. A study with primary cultures of rat epididymal epithelial cells confirmed the expression of CFTR and revealed its important role in regulating Cl^- secretion and epididymal fluid formation [51]. Several studies have demonstrated that the movement of water could be directly linked with the expression and activity of CFTR, through the establishment of cAMP that stimulates aqueous pore, or by interaction with aquaporins [52]. Aquaporins play a major role in water transport [53] across the epididymal epithelium and have been identified throughout the epididymis, including in the efferent ducts and vas deferens [54-60], but not necessarily in every region and in epithelial cells [57, 61, 62]. Therefore, besides being an ion channel, CFTR also regulates water permeability in some tissues or cells of the male reproductive tract. Hence, the abnormal functioning of CFTR may involve altered water transport and changes in HCO_3^- transport, resulting in alterations in the composition of the luminal tubular fluid [63]. Thereby, HCO_3^- is not only essential to ionic homeostasis, but it also plays a crucial role in the maintenance of pH along the male reproductive tract, particularly in epididymis, and still plays a central role in spermatozoa activation and capacitation.

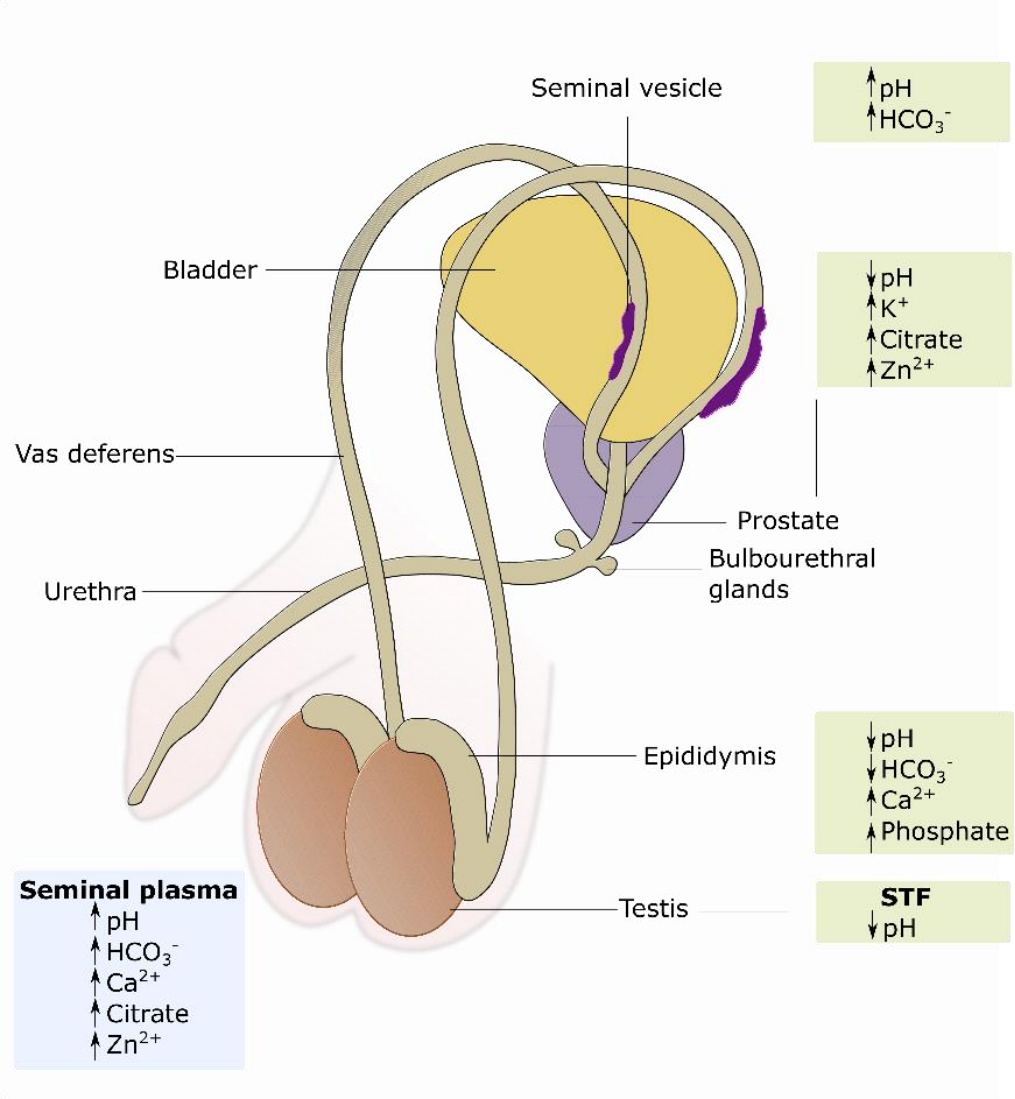


Figure 1.8 Representative image of the most significant electrolyte composition that constitutes the male reproductive tract fluid, which is strongly correlated with pH establishment. Seminiferous tubular fluid is produced into the testes and presents an acidic pH. In epididymis the pH is also acidic due to the low concentration of HCO_3^-, and high concentrations of Ca^{2+} and phosphate. Prostate gland produces a prostatic fluid with elevated concentrations of K^+, citrate and Zn^{2+} with acidic pH. Seminal vesicle is the main contributor with HCO_3^- for seminal plasma and consequently, the seminal liquid has a more alkaline pH. Seminal plasma is constituted by the addition of some fluids, those who have more relevance are the STF, prostatic and seminal fluid. Some of the ions contributing to an alkaline pH of seminal plasma are HCO_3^-, Ca^{2+}, citrate and Zn^{2+}. Abbreviations: STF, Seminiferous tubular fluid.

Luminal calcium and sperm function

Immature sperm formed in the seminiferous tubules of the testes become mature and motile in the epididymis. Sperm motility has been shown to be a good predictor of human male fertility *in vivo* and *in vitro* [64], being controlled by various parameters, such as Ca^{2+} and HCO_3^- concentrations in luminal fluid. Both Ca^{2+} and HCO_3^- concentrations are important components of the luminal epididymal fluid that could be important for sperm capacitation and motility

(Figure 1.8). It has been suggested that Ca^{2+}-binding proteins in sperm membrane directly stimulate the contractile apparatus of the sperm flagella [65]. Extracellular Ca^{2+} in the tail portion of the epididymis is required for motility in epididymal sperm, and Ca^{2+} is directly correlated with activated and hyperactivated sperm motility [66]. The Ca^{2+} concentration in epididymal fluid controls intracellular cAMP levels of spermatozoa through the regulation of sAC. This will lead to protein phosphorylation, which is the key to understand the initial activation of motility during epididymal transit [36]. Besides the regulation of sAC, other Ca^{2+} pathways independent of cAMP and protein kinase A (PKA) phosphorylation are involved, such as the calmodulin pathway [67].

Several channels that contribute to the control of membrane permeability to Ca^{2+} in the sperm membrane have been identified. The luminal Ca^{2+} is mainly absorbed by the membrane Ca^{2+} channel, transient receptor potential (TRP channels), which consist of six transmembrane helices and intracellular N- and C-terminal [68]. Two ubiquitous Ca^{2+} sensor calmodulin (CaM) binding sites have been identified so far in TRP channels [68, 69]. CaM is a constitutive or dissociable Ca^{2+}-sensing subunit for a large variety of ion channels, including TRP family channels. Although, at the moment not much is known about the functional role of the CaM molecules bound to a single functional channel [69]. For numerous TRP channels binding of CaM to the C-terminal intracellular part has been described to be essential for Ca^{2+}-dependent inactivation [70, 71], but very few details about the molecular mechanisms are presented [72]. Luminal variations in Ca^{2+} concentrations on the fluid that surrounds sperm during epididymal transit were detected. Between the caput and cauda epididymis, luminal Ca^{2+} concentration decreases from 0.8 to 0.3 mM in the rat epididymis (Table 1.2) [1, 23]. The luminal Ca^{2+} is mainly absorbed by the TRPV6 channel. In the end of TRPV6 structure there are approximately 30 amino acids corresponding to the CaM binding sites, and Ca^{2+}-dependent channel inactivation is enabled by CaM binding to this fragment [70]. Recent studies showed that there is a disruption of Ca^{2+} absorption by the epididymal epithelium when Trpv6 gene is deleted, resulting in increased Ca^{2+} concentrations in the caudal epididymal fluid [73]. When Ca^{2+} concentration is altered in rat epididymal fluid, is observed a partial loss of sperm motility and fertilization capacity [73]. It was reported that intracellular Ca^{2+} levels are six times higher in caput spermatozoa than in caudal spermatozoa, and the rate of Ca^{2+} uptake in caput spermatozoa seems to be about three times higher than that in caudal spermatozoa [74]. At least some of the effects of Ca^{2+} on sperm motility are possibly achieved through CaM, since CaM inhibition decreases sperm motility [74, 75]. However, the effects of Ca^{2+} on sAC are not dependent of CaM [67], which suggests that Ca^{2+} affects sperm motility via distinct pathways. Hence there are at last two different Ca^{2+} activated pathways in sperm: one that is dependent of CaM and one that is not.

In brief, CaM can regulate some cellular functions and control the intracellular concentrations of Ca^{2+} and cAMP. It has been suggested that in cells with endocrine activity, the mechanisms of Ca^{2+}-CaM are similar to the events that take place between steroid hormones and their receptor proteins in the cytoplasm [76]. Ca^{2+}/CaM is involved in multiple functions in sperm, including motility, capacitation and the acrosome reaction [75, 77]. CaM is located in the principal piece of the flagellum, where the main proteins directly involved in the regulation of motility are found. CaM inhibitors decrease sperm motility, but this inhibition was largely reversed by stimulation of PKA, but only when substrates for oxidative respiration (lactate and pyruvate) were present in the medium. In addition, if there is substrate for glycolysis (glucose) in the medium, but there is no lactate and pyruvate, increased intracellular cAMP cannot restore sperm motility. This suggests that CaM can be implicated in the regulation of glycolysis [78]. Therefore, the inhibition of CaM indirectly inhibits glycolysis and consequently sperm motility.

Ca^{2+} can also be directly linked to flagellar function through regulation of the atypical sAC, which generates cAMP to activate PKA. The sAC is required for sperm motility, and is different from the transmembrane adenylyl cyclases, since sAC is the unique sensitive to both HCO_3^- and Ca^{2+} [79]. sAC activity is molecularly distinct from transmembrane AC since its activity is dependent on the presence of Mn^{2+}, and it is insensitive to forskolin (which is known to increase cAMP levels in most cell types) and G protein regulation. The purified sAC exhibited 10-fold lower affinity for ATP relative to transmembrane AC (transmembrane AC Km for ATP-Mn^{2+} is approximately 100 μM and purified rat testes sAC Km for ATP-Mn^{2+} is approximately 1 mM) [80]. There is a synergistic interaction between HCO_3^- and Ca^{2+}, where HCO_3^- function to increase the Vmax (maximum velocity or rate at which the enzyme catalyzed a reaction) of enzyme, whereas Ca^{2+} increases its affinity for substrate ATP-Mn^{2+} [80]. sAC activity can be responsive to the elevated Ca^{2+} concentrations established during acrosome reaction and sperm motility, or sAC can be located close to the Ca^{2+} channel, where it may mediate the cAMP regulation of channel opening [81]. The PKA, the classic cAMP target, catalyzes the phosphorylation of several flagellar proteins, thus regulating sperm motility. The cAMP-PKA signaling pathway also induces phosphorylation of various proteins required for the capacitation response [82].

Electrolytes, phosphatases and seminal plasma composition

Seminal plasma is a fluid that originates from the testes, epididymis, prostate, and seminal vesicles secretions. As already described, a part of STF goes to the epididymis with spermatozoa, thus joining the epididymal fluid. Seminal plasma contains fluids originated by epididymal fluids that joins the fluid released by male accessory glands, prostatic fluid and the

one produced by seminal vesicles. Human seminal plasma contains calcium, citrate, zinc and magnesium in higher concentrations than in other body fluids [83] (Figure 1.8). The comparison of these electrolytes in prostatic and seminal fluid confirms that most of them are originated from the prostate. Prostate is the major source of seminal K^+ (66.8 mM by prostate and 27.2 mM by seminal vesicles) [83] (Figure 1.8). Less attention has been given to Na^+ and Cl^- that are also secreted by prostate gland and seminal vesicles [84]. While the concentrations of Cl^- are identical in prostatic and seminal fluid, Na^+ is slightly lower in seminal fluid, which may show that both have similar contributions in the two fluids [83]. The pH of the fluids is strongly correlated with electrolytic composition. The pH in seminal plasma is maintained around 7.5, essentially by HCO_3^- produced by accessory reproductive glands [24, 85]. The regulation of HCO_3^- levels in seminal plasma is achieved by the enzymatic activity of cytosolic carbonic anhydrase isoenzymes, secreted by seminal vesicle, prostate and bulbourethral gland [85]. It has been shown that citrate, zinc and pH (6.2 in prostate fluid) (Figure 1.8) in prostatic secretion are correlated to secretory enzyme activities, such as acid phosphatases, aminopeptidases and ATPases [86]. Prostatic acid phosphatase (PAP) is the essential phosphatase that degrades lysophosphatidic acid (LPA) in human seminal plasma [87]. LPA is a phospholipid that activates specific G protein coupled receptors to induce multiple cellular responses [88]. The expression of LPA in seminal plasma and sperm is high, but its physiological role is not known [87]. Alkaline phosphatase (AP) is another enzyme reported in seminal plasma from numerous species. AP catalyzes the hydrolysis of organic phosphate at basic pH values [89]. The physiological role of these enzymes is speculative in male reproductive tract, but they have been suggested to have an essential role in the active transport of substances across membranes. It is known that there are considerable variations among the AP activities of seminal plasma among different species. Human semen is relatively poor in AP compared with other species, such as in bull semen. There is an inverse relationship between the amounts of AP ejaculated and serum AP activity [90]. Seminal plasma contains prostasomes, which are vesicles secreted by prostate gland that contain cholesterol, sphingomyelin, Ca^{2+} and some other enzymes [91]. It is known that prostasomes fuse to sperm at slightly acidic pH values and that this phenomenon transfers lipid and protein, modifying the properties of sperm membranes [92]. Prostasomes improve sperm motility under conditions of low pH, which can affect the sperm competition in the vagina that tends to be acidic [3]. Seminal plasma has a high buffering capacity that is essential for sperm motility and fertilizing ability in environments with different ionic compositions and different pH.

Concluding remarks

It is evident that the maintenance of a proper fluid environment for spermatozoa storage and maturation depends on the regulation of absorption/reabsportion and secretion of electrolytes and fluids along the male reproductive tract, with greater emphasis on the processes that occur in epididymal duct. Significant progress has been made in our understanding about the composition of seminal plasma and fluids secreted by accessory glands, providing new insights that ultimately could improve human reproductive health. Given the general concern regarding a putative decline in male fertility, it seems imperative that seminal plasma alterations should become an area of intense future research with proper funds.

References

1. BP, S., M. S, and B. DE, Anatomy, Vasculature, and Innervation of the Male Reproductive Tract. In: The Physiology of Reproduction, in International journal of biochemistry, E. Knobil and J. Neill, Editors. 1994, Raven Press. p. 1063-1175.
2. Poiani, A., 2006, Complexity of seminal fluid: a review. Behavioral Ecology and Sociobiology, 60(3): p. 289-310.
3. Arienti, G., et al., 1999, The motility of human spermatozoa as influenced by prostasomes at various pH levels. Biology of the Cell, 91(1): p. 51-54.
4. Pastor-Soler, N., C. Piétrement, and S. Breton, 2005, Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. Physiology, 20(6): p. 417-428.
5. Bernardino, R.L., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current medicinal chemistry, 20(32): p. 4037-4049.
6. Cornwall, G.A., 2009, New insights into epididymal biology and function. Human reproduction update, 15(2): p. 213-227.
7. Rodríguez, C.M. and B.T. Hinton, The testicular and epididymal luminal fluid microenvironment, in Introduction to Mammalian Reproduction. 2003, Springer. p. 61-77.
8. Arrotéia, K.F., et al., The epididymis: embryology, structure, function and its role in fertilization and infertility, in Embryology - Updates and Highlights on Classic Topics, L.A.V. Pereira, Editor. 2012, INTECH Open Access Publisher. p. 41-66.
9. Dacheux, J.-L. and F. Dacheux, Protein secretion in the epididymis, in The Epididymis: From Molecules to Clinical Practice. 2002, Springer. p. 151-168.
10. Verjans, H., et al., 1974, Effect of oestradiol benzoate on pituitary and testis function in the normal adult male rat. Acta endocrinologica, 77(4): p. 636-642.
11. Keller, R., 2002, Shaping the vertebrate body plan by polarized embryonic cell movements. Science, 298(5600): p. 1950-1954.

12. Di Giovanni, V., et al., 2011, Alk3 controls nephron number and androgen production via lineage-specific effects in intermediate mesoderm. Development, 138(13): p. 2717-2727.
13. Farr, C. and L. Ellis, 1980, In-vitro contractility of rat seminiferous tubules in response to prostaglandins, cyclic GMP, testosterone and 2, 4′-dibromoacetophenone. Journal of reproduction and fertility, 58(1): p. 37-42.
14. Hinton, B. and B. Setchell, Fluid secretion and movement. In: The Sertoli Cell, Russell L., Griswold M., Editors. 1993, Cache River Press, Clearwater,12: p. 249-267.
15. Mann, T. and C. Lutwak-Mann, Testis and testicular semen, in Male Reproductive Function and Semen. 1981, Springer. p. 83-138.
16. Elzanaty, S., et al., 2002, The impact of epididymal and accessory sex gland function on sperm motility. Human Reproduction, 17(11): p. 2904-2911.
17. Turner, T., Necessity's potion: inorganic ions and small organic molecules in the epididymal lumen, in The Epididymis: From Molecules to Clinical Practice. 2002, Springer. p. 131-150.
18. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. The Journal of membrane biology, 236(2): p. 215-224.
19. Fisher, D., 2002, New light shed on fluid formation in the seminiferous tubules of the rat. The Journal of physiology, 542(2): p. 445-452.
20. Henning, R. and J. Young, 1971, Electrolyte transport in the seminiferous tubules of the rat studied by the stopped-flow microperfusion technique. Experientia, 27(9): p. 1037-1039.
21. Levine, N. and D.J. Marsh, 1975, Micropuncture study of the fluid composition of sertoli cell-only'seminiferous tubules in rats. Journal of reproduction and fertility, 43(3): p. 547-549.
22. Tuck, R., et al., 1970, The composition of fluid collected by micropuncture and catheterization from the seminiferous tubules and rete testis of rats. Pflügers Archiv: European journal of physiology, 318(3): p. 225-243.
23. Jenkins, A.D., C.P. Lechene, and S.S. Howards, 1980, Concentrations of seven elements in the intraluminal fluids of the rat seminiferous tubules, rete testis, and epididymis. Biology of Reproduction, 23(5): p. 981-987.
24. Levine, N. and D.J. Marsh, 1971, Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. The Journal of physiology, 213(3): p. 557-570.
25. Tao, L., J. Zupp, and B. Setchell, 2000, Effect of efferent duct ligation on the function of the blood-testis barrier in rats. Journal of reproduction and fertility, 120(1): p. 13-18.
26. Zhou, Q., et al., 2001, Estrogen action and male fertility: roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. Proceedings of the National Academy of Sciences, 98(24): p. 14132-14137.

27. Wong, P. and C. Yeung, 1976, Inhibition by amiloride of sodium-dependent fluid reabsorption in the rat isolated caudal epididymis. British journal of pharmacology, 58(4): p. 529-531.
28. Wong, Y., P. Wong, and C. Yeung, 1978, Ultrastructural correlation of water reabsorption in isolated rat cauda epididymidis. Experientia, 34(4): p. 485-487.
29. Wong, P., C. Au, and H. Ngai, 1978, Electrolyte and water transport in rat epididymis; its possible role in sperm maturation. International Journal of Andrology, 1(s2b): p. 608-628.
30. Levine, N. and H. Kelly, 1978, Measurement of pH in the rat epididymis in vivo. Journal of reproduction and fertility, 52(2): p. 333-335.
31. Wong, P. and W. Lee, 1983, Potassium movement during sodium-induced motility initiation in the rat caudal epididymal spermatozoa. Biology of Reproduction, 28(1): p. 206-212.
32. Liu, Y., D.-K. Wang, and L.-M. Chen, 2012, The physiology of bicarbonate transporters in mammalian reproduction. Biology of Reproduction, 86(4): p. 99.
33. Hermo, L., et al., 2005, Region-and cell-specific differences in the distribution of carbonic anhydrases II, III, XII, and XIV in the adult rat epididymis. Journal of Histochemistry & Cytochemistry, 53(6): p. 699-713.
34. Okamura, N., Y. Tajima, and Y. Sugita, 1988, Decrease in bicarbonate transport activities during epididymal maturation of porcine sperm. Biochemical and biophysical research communications, 157(3): p. 1280-1287.
35. Chen, Y., et al., 2000, Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science, 289(5479): p. 625-628.
36. Zippin, J.H., et al., 2003, Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. The FASEB Journal, 17(1): p. 82-84.
37. Dacheux, J.-L. and F. Dacheux, 2014, New insights into epididymal function in relation to sperm maturation. Reproduction, 147(2): p. R27-R42.
38. Boron, W.F., 2004, Regulation of intracellular pH. Advances in physiology education, 28(4): p. 160-179.
39. Oliveira, P., et al., 2009, Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. Journal of Membrane Biology, 227(1): p. 49-55.
40. Au, C. and P. Wong, 1980, Luminal acidification by the perfused rat cauda epididymidis. The Journal of physiology, 309: p. 419.
41. Breton, S., et al., 1996, Acidification of the male reproductive tract by a proton pumping (H+)-ATPase. Nature medicine, 2(4): p. 470-472.
42. Martins, A.D., et al., 2014, Physiology of na+/h+ exchangers in the male reproductive tract: relevance for male fertility. Biology of Reproduction, 91(1): p. 11.

43. Puskin, A., et al., 2000, Immunolocalization of NBC3 and NHE3 in the rat epididymis: colocalization of NBC3 and the vacuolar H+-ATPase. Journal of andrology, 21(5): p. 708-720.
44. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and cellular biochemistry, 408(1-2): p. 47-54.
45. Bernardino, R.L., et al., 2013, Effect of prediabetes on membrane bicarbonate transporters in testis and epididymis. The Journal of membrane biology, 246(12): p. 877-883.
46. Jensen, L.J., et al., 1999, Immunolocalization of AE2 anion exchanger in rat and mouse epididymis. Biology of Reproduction, 61(4): p. 973-980.
47. Rato, L., et al., 2013, High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. Andrology, 1(3): p. 495-504.
48. Hihnala, S., et al., 2006, Expression of SLC26A3, CFTR and NHE3 in the human male reproductive tract: role in male subfertility caused by congenital chloride diarrhoea. Molecular human reproduction, 12(2): p. 107-111.
49. Kujala, M., et al., 2007, Expression of ion transport-associated proteins in human efferent and epididymal ducts. Reproduction, 133(4): p. 775-784.
50. Schweinfest, C.W., et al., 1993, Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas. Proceedings of the National Academy of Sciences, 90(9): p. 4166-4170.
51. Tizzano, E.F., et al., 1994, Differential cellular expression of cystic fibrosis transmembrane regulator in human reproductive tissues. Clues for the infertility in patients with cystic fibrosis. The American journal of pathology, 144(5): p. 906.
52. Wong, P., 1998, CFTR gene and male fertility. Molecular human reproduction, 4(2): p. 107-110.
53. Schreiber, R., et al., 1997, Cystic fibrosis transmembrane conductance regulator activates water conductance in Xenopus oocytes. Pflügers Archiv: European journal of physiology, 434(6): p. 841-847.
54. Bernardino, R.L., et al., 2016, Hepatocyte and Sertoli Cell Aquaporins, Recent Advances and Research Trends. International Journal of Molecular Sciences, 17(7): p. E1096.
55. Brown, D., et al., 1993, Localization of the CHIP28 water channel in reabsorptive segments of the rat male reproductive tract. European journal of cell biology, 61(2): p. 264-273.
56. Fisher, J.S., et al., 1998, Immunoexpression of aquaporin-1 in the efferent ducts of the rat and marmoset monkey during development, its modulation by estrogens, and its possible role in fluid resorption 1. Endocrinology, 139(9): p. 3935-3945.
57. Elkjær, M.-L., et al., 2000, Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. Biochemical and biophysical research communications, 276(3): p. 1118-1128.

58. Pastor-Soler, N., et al., 2001, Aquaporin 9 expression along the male reproductive tract. Biology of Reproduction, 65(2): p. 384-393.
59. Badran, H.H. and L.S. Hermo, 2002, Expression and regulation of aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development. Journal of andrology, 23(3): p. 358-373.
60. Pastor-Soler, N., et al., 2002, Expression of aquaporin 9 in the adult rat epididymal epithelium is modulated by androgens. Biology of Reproduction, 66(6): p. 1716-1722.
61. Hermo, L., D. Krzeczunowicz, and R. Ruz, 2004, Cell specificity of aquaporins 0, 3, and 10 expressed in the testis, efferent ducts, and epididymis of adult rats. Journal of andrology, 25(4): p. 494-505.
62. Da Silva, N., et al., 2006, Segmental and cellular expression of aquaporins in the male excurrent duct. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1758(8): p. 1025-1033.
63. Hermo, L., et al., 2008, Membrane domain specificity in the spatial distribution of aquaporins 5, 7, 9, and 11 in efferent ducts and epididymis of rats. Journal of Histochemistry & Cytochemistry, 56(12): p. 1121-1135.
64. Alves, M.G., et al., 2015, CFTR regulation of Aquaporin-mediated water transport: a target in male fertility. Current drug targets, 16(9): p. 993-1006.
65. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.
66. Huang, H.-F., et al., 2006, Function of aquaporins in female and male reproductive systems. Human reproduction update, 12(6): p. 785-795.
67. Setchell, B., et al., 1969, Characteristics of testicular spermatozoa and the fluid which transports them into the epididymis. Biology of Reproduction, 1(Supplement 1): p. 40-66.
68. Ilio, K.Y. and R.A. Hess, 1994, Structure and function of the ductuli efferentes: a review. Microscopy research and technique, 29(6): p. 432-467.
69. Wong, P., et al., Formation of the epididymal fluid microenvironment, in The epididymis: from molecules to clinical practice, Robaire B. and Hinton B.T., Editors. 2002, Springer. p. 119-130.
70. Preston, G. and P. Agre, 1991, Molecular cloning of the red cell integral protein of Mr 28000: a member of an ancient channel family. Proceedings of the National Academy of Sciences USA, 88: p. 11110-11114.
71. Nicotina, P.A., et al., 2004, Immunohistology of aquaporin-1 and stem cell factor-receptor in human undescended testes. Pediatric surgery international, 20(4): p. 271-275.

72. Klein, C., M. Troedsson, and J. Rutllant, 2013, Region-Specific Expression of Aquaporin Subtypes in Equine Testis, Epididymis, and Ductus Deferens. The Anatomical Record, 296(7): p. 1115-1126.
73. Jesus, T.T., et al., 2014, Aquaporin-4 as a molecular partner of cystic fibrosis transmembrane conductance regulator in rat Sertoli cells. Biochemical and biophysical research communications, 446(4): p. 1017-1021.
74. Ishibashi, K., et al., 1997, Cloning and functional expression of a second new aquaporin abundantly expressed in testis. Biochemical and biophysical research communications, 237(3): p. 714-718.
75. Jesus, T.T., et al., 2014, Aquaporin-9 is expressed in rat Sertoli cells and interacts with the cystic fibrosis transmembrane conductance regulator. IUBMB life, 66(9): p. 639-644.
76. Yeung, C., et al., 2010, Aquaporins in the human testis and spermatozoa–identification, involvement in sperm volume regulation and clinical relevance. International Journal of Andrology, 33(4): p. 629-641.
77. Domeniconi, R.F., et al., 2008, Immunolocalization of aquaporins 1, 2 and 7 in rete testis, efferent ducts, epididymis and vas deferens of adult dog. Cell and tissue research, 332(2): p. 329-335.
78. Hermo, L. and C.E. Smith, 2011, Thirsty business: cell, region, and membrane specificity of aquaporins in the testis, efferent ducts, and epididymis and factors regulating their expression. Journal of andrology, 32(6): p. 565-575.
79. Hess, R., et al., 1997, Estrogen Receptor (p) Expression in the Excurrent Ducts of the Adult Male Rat Reproductive Tract. Journal of andrology, 18(6): p. 602-611.
80. Auger, J., et al., 1994, Sperm motility and fertilization. Contraception, fertilite, sexualite (1992), 22(5): p. 314-318.
81. Babcock, D.F., D.M. Stamerjohn, and T. Hutchinson, 1978, Calcium redistribution in individual cells correlated with ionophore action on motility. Journal of Experimental Zoology, 204(3): p. 391-399.
82. Morton, B.E., R. Sagadraca, and C. Fraser, 1978, Sperm motility within the mammalian epididymis: species variation and correlation with free calcium levels in epididymal plasma. Fertility and sterility, 29(6): p. 695-698.
83. Jaiswal, B.S. and M. Conti, 2003, Calcium regulation of the soluble adenylyl cyclase expressed in mammalian spermatozoa. Proceedings of the National Academy of Sciences, 100(19): p. 10676-10681.
84. Zhu, M.X., 2005, Multiple roles of calmodulin and other Ca2+-binding proteins in the functional regulation of TRP channels. Pflügers Archiv: European journal of physiology, 451(1): p. 105-115.

85. Saimi, Y. and C. Kung, 2002, Calmodulin as an ion channel subunit. Annual review of physiology, 64(1): p. 289-311.
86. Niemeyer, B.A., et al., 2001, Competitive regulation of CaT-like-mediated Ca2+ entry by protein kinase C and calmodulin. Proceedings of the National Academy of Sciences, 98(6): p. 3600-3605.
87. Lambers, T.T., et al., 2004, Regulation of the mouse epithelial Ca2+ channel TRPV6 by the Ca2+-sensor calmodulin. Journal of Biological Chemistry, 279(28): p. 28855-28861.
88. Gordon-Shaag, A., W.N. Zagotta, and S.E. Gordon, 2008, Mechanism of Ca2+-dependent desensitization in TRP channels. Channels, 2(2): p. 125-129.
89. Weissgerber, P., et al., 2012, Excision of Trpv6 gene leads to severe defects in epididymal Ca2+ absorption and male fertility much like single D541A pore mutation. Journal of Biological Chemistry, 287(22): p. 17930-17941.
90. White, D.R. and R.J. Aitken, 1989, Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete research, 22(2): p. 163-177.
91. Si, Y. and P. Olds-Clarke, 2000, Evidence for the involvement of calmodulin in mouse sperm capacitation. Biology of Reproduction, 62(5): p. 1231-1239.
92. Means, A.R. and J.R. Dedman, 1980, Calmodulin--an intracellular calcium receptor. Nature, 285(5760): p. 73-77.
93. Bendahmane, M., C. Lynch, and D.R. Tulsiani, 2001, Calmodulin signals capacitation and triggers the agonist-induced acrosome reaction in mouse spermatozoa. Archives of biochemistry and biophysics, 390(1): p. 1-8.
94. Schlingmann, K., et al., 2007, Calmodulin and CaMKII in the Sperm Principal Piece: Evidence for a Motility-Related Calcium/Calmodulin Pathway. Journal of andrology, 28(5): p. 706-716.
95. Esposito, G., et al., 2004, Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. Proceedings of the National Academy of Sciences of the United States of America, 101(9): p. 2993-2998.
96. Litvin, T.N., et al., 2003, Kinetic properties of “soluble” adenylyl cyclase synergism between calcium and bicarbonate. Journal of Biological Chemistry, 278(18): p. 15922-15926.
97. Darszon, A., et al., 1999, Ion channels in sperm physiology. Physiological Reviews, 79(2): p. 481-510.
98. Kaupp, U.B. and I. Weyand, 2000, A universal bicarbonate sensor. Science, 289(5479): p. 559-560.

99. Kavanagh, J., 1985, Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. Journal of reproduction and fertility, 75(1): p. 35-41.
100. Knobil, E. and J.D. Neill, Knobil and Neill's physiology of reproduction. Vol. 2. 2006: Gulf Professional Publishing.
101. McNeal, J.E., 1988, Normal histology of the prostate. The American journal of surgical pathology, 12(8): p. 619-633.
102. Morrissey, C., J.M. Fitzpatrick, and R.W.G. Watson, 2005, Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. Clinical science, 108(1): p. 1-11.
103. Wang, Y., et al., 2001, Cell differentiation lineage in the prostate. Differentiation, 68(4-5): p. 270-279.
104. Pritchard, C.C. and P.S. Nelson, 2008, Gene expression profiling in the developing prostate. Differentiation, 76(6): p. 624-640.
105. Lau, K.-M., et al., 2000, Expression of estrogen receptor (ER)-α and ER-β in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. Cancer research, 60(12): p. 3175-3182.
106. Humphrey, G. and T. Mann, 1949, Studies on the metabolism of semen. 5. Citric acid in semen. Biochemical Journal, 44(1): p. 97.
107. Huggins, C., W.W. Scott, and J.H. Heinen, 1942, Chemical composition of human semen and of the secretions of the prostate and seminal vesicles. American Journal of Physiology--Legacy Content, 136(3): p. 467-473.
108. Asari, M., et al., 1996, Immunohistolocalization of the carbonic anhydrase isoenzymes (CA-I, CA-II, and CA-III) in the reproductive tract of male horses. American journal of veterinary research, 57(4): p. 439-443.
109. Kavanagh, J. and C. Darby, 1982, The interrelationships between acid phosphatase, aminopeptidase, diamine oxidase, citric acid, β-glucuronidase, pH and zinc in human prostatic fluid. International Journal of Andrology, 5(5): p. 503-512.
110. Tanaka, M., et al., 2004, Prostatic acid phosphatase degrades lysophosphatidic acid in seminal plasma. FEBS letters, 571(1-3): p. 197-204.
111. Moolenaar, W.H., et al., 1997, Lysophosphatidic acid: G-protein signalling and cellular responses. Current opinion in cell biology, 9(2): p. 168-173.
112. Harris, H., 1990, The human alkaline phosphatases: what we know and what we don't know. Clinica Chimica Acta, 186(2): p. 133-150.
113. McComb, R.B., G.N. Bowers Jr, and S. Posen, Alkaline phosphatase. 2013, New York: Springer Science & Business Media.

114. Kravets, F.G., et al., 2000, Prostasomes: current concepts. The Prostate, 43(3): p. 169-174.
115. Carlini, E., et al., 1997, Fusion of sperm with prostasomes: effects on membrane fluidity. Archives of biochemistry and biophysics, 343(1): p. 6-12.

Chapter 2

Objectives

Objectives

Infertility affects millions of couples worldwide and almost one third of the cases results from a defect in male fertility. Spermatozoa production and maturation is a complex process during which the establishment of the adequate environments throughout the male reproductive tract is a key factor. The testicular compartmentalization established by the Sertoli cells (SCs), which form the blood-testis barrier (BTB) or Sertoli cell barrier, creates a controlled luminal milieu within this organ. This millieu, the seminiferous tubular fluid, has a specific composition being distinct from interstitial fluid and plasma, and providing the milieu for spermatogenesis occurrence, spermatozoa development and transport. The SCs directly control the seminiferous tubular fluid composition by regulating the selective passage/absorption of ions and water. In the last decades, estrogens emerged as essential role for male reproduction being able to modulate key events determining the reproductive potential of males. Herein, we hypothesize that sex steroid hormones can modulate intratubular fluid composition by regulating the ion and water transport, with a particular action in SCs.

We aimed to study important mechanisms in the transport of ions and metabolites in the function of SCs. We also assessed the effects of hormonal dysregulation on these transport mechanisms. To better understand the role of estrogens on the control of testicular membrane transport of ions and water, we focused on studying the impact of physiological and non-physiological levels of those hormones on the expression and function of specific membrane transporters. Additionally, we aimed to relate clinical reproductive disorders associated with increased levels of estrogens (Klinefelter syndrome, idiopathic infertility) with the dysregulation of membrane transport mechanisms. Finally, we aimed to establish the link between Klinefelter syndrome and/or idiopathic infertility with high estrogen levels and membrane transport (dys)regulation. The principal goals of this project were the following:

1. Bicarbonate ion is essential for ionic regulation of body fluids. In testis, namely in SCs, little is known about its production and role of carbonic anhydrases (CAs), a group of enzymes that catalyses the reversible hydration of CO_2, and serve to accelerate the (normally slow) equilibration between CO_2 and HCO_3^-. Thus, it is of great relevance to understand the role of CAs, in the cells of the male reproductive tract that are responsible for the homeostasis of fluids. Herein, we aimed to evaluate the effects of general and selective inhibition of CAs in the physiology and metabolism of human SCs (hSCs). The hSCs primary cultures were isolated from testicular biopsies of men with conserved spermatogenesis, selected from patients with anejaculation, vasectomy or traumatic section of the vas deferens.

2. The microenvironment created by SCs influences the development of germ cells and all accountable processes for the formation of viable sperm. In male reproductive tract, SCs are major targets for the hormonal signalling that regulates spermatogenesis. CAs play an essential role in the formation of HCO_3^- and HCO_3^- membrane transporters are amongst the most relevant epithelial ion transporters present in the male reproductive tract. However, their involvement on ionic regulation of tubular fluid secretion and the hormonal modulation of their function remain unclear. We aimed to determine the role of estrogens in the modulation of intratubular fluid composition by regulating the expression and function of those HCO_3^- membrane transporters in hSCs.
3. Glycerol and water are essential for the normal occurrence of spermatogenesis and maintenance of BTB. Since aquaglyceroporins (AQPs) are responsible for the transport of glycerol and its homeostasis is vital for male reproductive health, we aimed to evaluate the impact of estrogens on AQPs expression and consequently on glycerol permeability in SCs.
4. Klinefelter syndrome is the most common sex chromosome abnormality in men. Adults with Klinefelter syndrome present small firm testes, gynaecomastia and hormonal dysregulation. Serum levels of testosterone are often in the lower half of the reference range for healthy male individuals, while serum estradiol (E2) levels are high at the beginning of puberty and remain high throughout the onset of the adult life. The estrogenic actions described are mediated by the estrogen receptors alpha (ERα), estrogen receptors beta (ERβ) and G protein-coupled estrogen receptor (GPR30). Yet, little is known about the expression of these E2 receptors in testicular tissue of Klinefelter syndrome men, despite the recognized alterations on the E2 serum levels. Hence, we aimed to evaluate the expression levels of these E2 receptors in the testicular tissue of individuals with Klinefelter syndrome. We would also like to correlate the possible alterations in the membrane transporters with E2 levels in Klinefelter syndrome men.

Chapter 3

Establishment of Primary culture of Sertoli Cells

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Establishment of Primary culture of Sertoli Cells

Abstract

The successful isolation and culture of Sertoli cells depends on a series of delicate processes of mechanical isolation and enzymatic digestion of the testicular tissue, taking advantage of an array of enzymes (such as DNAse, collagenase, and pancreatin) in order to digest the extracellular matrix components. The complexity of these processes may present some differences depending on the origin of the testicular sample (whole tissue or biopsy) and of the species in question. Rat and mouse Sertoli cells are obtained by a similar protocol, whereas bovine and human Sertoli cells require a more extensive mechanical and enzymatic processing.

Introduction

In vitro experiments with living cells can be performed with cell derived from immortalized lines or from primary cultures. The studies available on the regulation and function of Sertoli cells are derived mainly from in vitro primary cultured Sertoli cells isolated from the testes [1-3]. This approach has some advantages and some disadvantages, being that the major drawbacks is the time spent to isolate and culture the cells and the continuous necessity to use fresh tissue [4].

The rat and mouse remain the preferred animals for this experimental procedure [5]. For primary cultures, Sertoli cells should be obtained from prepubertal animals [6], since proliferation of Sertoli cells occurs essentially before the birth and falls steadily after parturition [7]. The use of prepubertal testes favors a better adaptation of the Sertoli cells in vitro conditions, and the contamination by germ cells is lower because at this stage there are still few germ cells in seminiferous tubules [8]. It is known that inter-Sertoli junctions develop before the onset of puberty period [9,10]. Rats [11] and mouse [12] with 20 days of age present a completed formation of the blood-testis barrier (BTB), as seen in adult animals, and have not yet started the spermatogenesis [11]. Hence, primary cultures of rat and mouse Sertoli cells are often made with animals about 20-22 days old. The bovines also are commonly used for in vitro studies with Sertoli cells [13], due to the high amount of testicular tissue per animal. Bulls reach the puberty at about 18 months [14], and for that reason Sertoli cell cultures must be made with tissue from animals before they reach this age. There are also several studies with primary cultures of human Sertoli cells, however, in this case, the vast majority are obtained from testicular biopsies, rather than the whole testicular tissue [15,16].

In this protocol, the tissue dissociation conditions are selected to minimize damage to Sertoli cells and preserve their physiological and morphological characteristics. This procedure is described for obtaining essentially Sertoli cell cultures from rat and mouse and alternatives are given for the protocol to obtain bovine and human Sertoli cell clustures, with the modifications on the enzymatic and mechanical procedures.

Materials

This procedure must be done in a sterile environment and ultrapure water should be used to prepare all solutions, which should be kept at 4ºC (unless indicated otherwise) (see Note 1).

- **Cell Culture**

1. Hank´s Balanced Salt Solution (HBSS): 140 mM sodium chloride, 5 mM potassium chloride, 0.44 mM potassium phosphate monobasic anhydrous, 0.34 mM sodium phosphate dibasic, 6 mM D-glucose and 4 mM sodium hydrogen carbonate. Weigh all the reagents into a 1 L graduated cylinder and dissolve in 900 mL of water (see Note 2). The pH should be adjusted to 7.4, using concentrated solutions of hydrochloric acid or/and sodium hydroxide. Adjust the volume to 1 L. Sterilize by filtering through a 0.2 µm mixed cellulose ester membrane filter using a vacuum filtration unit in the laminar flow chamber in a sterile environment, and store in a sterile bottle at 4 ºC.

2. Phosphate-buffered Saline Solution (PBS): 140 mM sodium chloride, 3 mM potassium chloride, 10 mM sodium phosphate dibasic and 1.8 mM potassium phosphate monobasic anhydrous. Weigh all reagents into a 1 L graduated cylinder and dissolve in 900 mL of water. The simplest method for making PBS is to prepare a 10×PBS stock solution (1.4 M sodium chloride, 0.03 M potassium chloride, 0.1 M sodium phosphate dibasic and 0.18 M potassium phosphate monobasic anhydrous) solution in 1 L of water. To prepare a working solution of PBS add 100 mL of 10×PBS stock to 900 mL water. If necessary, the pH of the solution should be adjusted to 7.4, using concentrated solutions of hydrochloric acid or/and sodium hydroxide. Adjust the volume to 1 L. The PBS should be filtered through a 0.2 µm mixed cellulose ester membrane filter using a vacuum filtration unit in the laminar flow chamber in a sterile environment, and store in a sterile bottle at 4 ºC (see Note 3).

- **Enzyme solutions**

1. Glycine solution: 100 mL HBSS, 50 U/mL Penicillin and 50 mg/mL Streptomycin, 1 M Glycine, 2 mM EDTA and 0.002% trypsin inhibitor. Adjust the pH to 7.2, using concentrated solutions of hydrochloric acid or/and sodium hydroxide and filter to sterilize.

2. DNAse solution: Add 1 mg DNAse (250 U) to 20 mL Glycine solution. Filter to sterilize.

3. Collagenase solution 1: 20 mL HBSS, 1 mg DNAse (250 U) and 4.5 mg Collagenase type I. Filter to sterilize.
4. Collagenase solution 2: 12.5 mL HBSS, 3.5 mg collagenase type I, 3.5 mg DNAse (250 U). Filter to sterilize.
5. Pancreatin Solution: 10 mL HBSS, 2.5 mg Pancreatin and 2.5 mg DNAse (250 U). Filter to sterilize.

- **Culture medium**

1. For the preparation of culture medium, you should start by adding Dulbecco's Modified Eagle's Medium - DMEM (with glucose and L-glutamine) with Nutrient Mixture F-12 Ham (with glucose and L-glutamine) and dissolve in 900 mL of water according to the manufacturer's recommendations (see Note 4). After add 14 mM sodium hydrogen carbonate and 15 mM HEPES.
2. If low-glucose DMEM medium is used, it should be supplemented with D-Glucose until reaching a final concentration of 18 mM.
3. The pH of the medium should be adjusted to 7.4, using concentrated solutions of hydrochloric acid or/and sodium hydroxide. Adjust the volume to 1 L.
4. This solution should be filtered through a 0.2 µm mixed cellulose ester membrane filter using a vacuum filtration unit in the laminar flow chamber in a sterile environment,
5. Add to the sterile medium 50 mL Fetal bovine serum (FBS) (10%), 50 µg/mL Gentamicin, 50 U/mL Penicillin and 50mg/mL Streptomycin (all these reagents should be sterile) (see Note 5). Store in a sterile bottle at 4 ºC.

Methods

These methods are essentially for the isolation of rat and mouse Sertoli cells. However, some alternatives will be given to the protocol for bovine and human Sertoli cell cultures (see Note 6) (Figure 3.1). The tissue dissociation conditions are chosen to minimize damage to Sertoli cells throughout the sequential enzymatic and mechanical. First, soak the outside of the skin of the euthanized animal in ethanol 70% and remove the testes as aseptically as possible. After removal of the testes, the all protocol should be done inside a laminar chamber, in a sterile environment.

- **Mechanical dispersion**

1. Place the right and left testis of the same animal (if rat or mouse) in a 50 mL conical tube with 20 mL HBSS a 4 ºC.
2. Wash twice with HBSS at 4 ºC, shaking gently.
3. Place the testes in dish petri a 4 ºC in sterile environment. With the support of tweezers and a scissor remove the tunica albuginea, as well as the testicular blood vessels (see Note 7).
4. Wash twice with HBSS a 4 ºC, shaking gently.
For bovine testis only: The tissue should be cut into small pieces. An amount of tissue corresponding to 1 g should be placed in 25 mL of HBSS and shaken vigorously during 1 minute. The tissue should be let to settle on ice for 5 minutes and the supernatant removed. Repeat this process 3 times (see Note 8).
5. Wash once with glycine solution (see Note 9).
For bovine testis only: this and the subsequent step are not to be taken in account when performing bovine Sertoli cells cultures.
6. Place the testicular tissue in a petri dish and carefully disperse the seminiferous tubules with the help of tweezers.

- **Enzymatic digestion of rat and mouse testicular tissue**

1. Place the seminiferous tubules in 50 mL conical tubule with 20 mL DNAse solution in a sterile environment, and incubate for 10 minutes at room temperature.
2. Gently pipette the tubules through a large-bore glass Pasteur pipette. This procedure allows the release of the cells of the testicular interstitial tissue, Leidyg and peritubular cells. Pellet the loosen tubules by gravity and discard the supernatant.
3. Wash 3 times with HBSS, allowing tubules to settle by gravity. Pellet the loosen tubules by gravity and discard the supernatant.
4. Resupend the pellet obtained at the last wash in 20 mL Collagenase solution 1 and incubate at room temperature during 20 minutes (see Note 10).
5. Centrifuge at 300xg during 3 minutes at room temperature and discard the supernatant.
6. Wash 3 times with HBSS by centrifuging at 300xg during 3 minutes, discard the supernatant.
7. Resuspend the pellet in culture medium and centrifuge at 300xg during 3 minutes.
8. Resuspend the resulting pellet in 5 mL culture medium and force the remaining tissue through a 20G needle, in order to disaggregate the cell clusters (see Note 11).

- **Enzymatic digestion of bovine testicular tissue**

1. Resuspend the pellet obtained in the last wash in 12.5 mL Collagenase solution 2 and shake (160 oscillations/minute) during approximately 30 minutes at 32ºC.

2. Wash the digested tissue with HBSS and discard the aggregate formed saving the intact tissue and the washing medium.

3. Centrifuge the resulting suspension (intact tissue and washing medium) at 250xg during 3 minutes and wash twice in HBSS.

4. Resuspend the pellet obtained in 10 mL Pancreatin solution and shake (160 oscillations / minute) during 15-25 minutes at 32ºC, until a new aggregate is formed (see Note 12).

5. Reserve the cellular suspension and wash the aggregate with 10 mL of HBSS. Add the washing solution to the previous cellular suspension and discard the aggregate.

6. Add 0.2 ml of FBS to the suspension and centrifuge at 250xg during 3.5 minutes (see Note 13).

7. Wash the pellet 3 times with 10 mL of HBSS by centrifuging at 250xg during 3.5 minutes.

8. Resuspend the pellet in 5 mL of HBSS with 5% FBS and put at 4ºC during 5 minutes.

9. Centrifuge at 250xg during 4 minutes at room temperature and discard supernatant.

10. Wash the pellet with 10 mL of HBSS and centrifuge 250xg during 4 minutes (see Note 14).

11. Resuspend the resulting pellet in 5 mL of Culture medium and force through a 20G needle, in order to disaggregate the cell clusters.

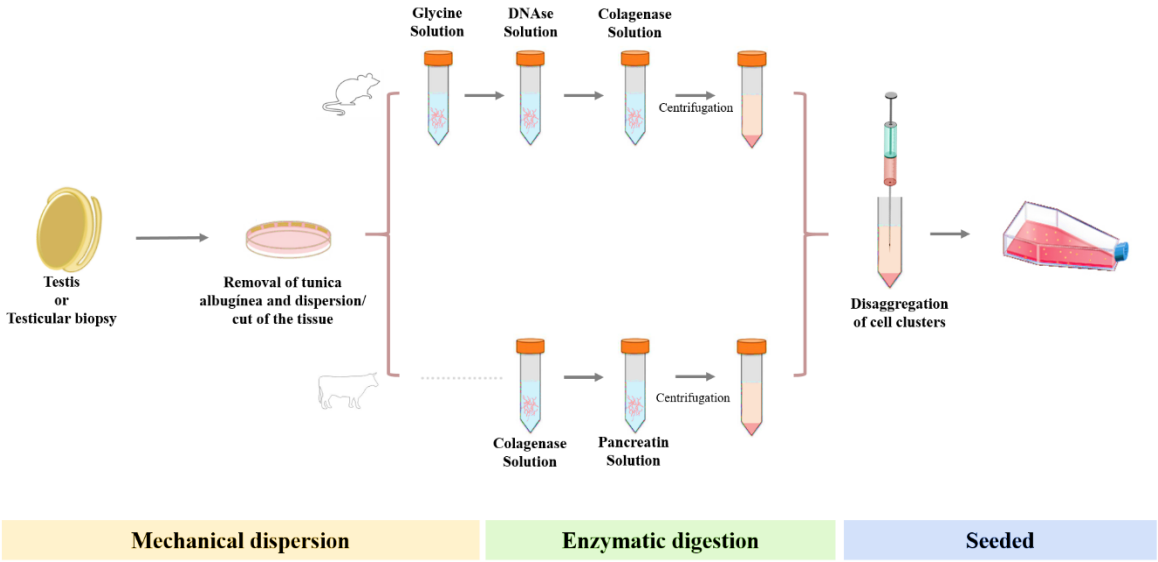

Figure 3.1 Simplified schematic representation of methodological approach of primary culture of Sertoli cells. The testis (rat, mouse or bovine) or testicular biopsy (bovine or human) is collected and the tunica albuginea is removed and then the tissue is fragmented. Depending on the origin of the sample, the tissue may undergo osmotic shock with the glycine solution and then enzymatic digestion with a solution of DNAse and collagenase, this protocol in the case of rat and mouse. In case of the bovine or human sample the enzymatic digestion is made with collagenase and pancreatin solution. Before seeding the cells in the flask, the pellet is passed through a needle for disaggregation of the clusters.

- **Seeding**

1. Seed the cells in tissue culture flasks and incubate at 33 ºC (5% CO_2).
2. Cell cultures should be allowed to stand undisturbed for 2 days, and thereafter should be inspected under the microscope. The culture medium should be replaced as frequently as necessary (see Note 15).

- **Maintenance of cell cultures**

1. Observe the cells using an invested optical microscope (see Note 16).
2. Discard the culture flask medium under sterile conditions.
3. Wash 3 times with sterile PBS (33-37 °C) (see Note 17).
4. Replace with fresh culture medium preheated (33-37 °C) and place the cell in the incubator.

Notes

1. All solutions should be prepared the day before the Sertoli cells culture.
2. Add about 500 mL water to a 1 L graduated cylinder and add one chemical at a time, while shaking, to dissolve the salts easier. The Sodium hydrogen carbonate should be the last to be added when the salts are dissolved, for not to precipitate.
3. This solution can be purchased already prepared and sterile.
4. DMEM and F12 can be acquired in powder or in liquid form. If acquired as powder, consult the manufacturer's instructions in order to know in which volume of water the powder must be dissolved.
5. Antimycotics may also be added to the culture medium (e.g. 0.5 mg/mL Amphotericin B) but these not essential for the cell culture.
6. For primary cultures of human Sertoli cells, if using small testicular samples (biopsies), perform only steps 9 and 10 of section 2.3, and proceed to section 2.4. If using a large sample of testicular tissue or the entire testis, you should follow the protocol described for the isolation of bovine Sertoli cells.
7. To maintain the temperature at 4 ºC during the mechanical dispersion, place an ice pack under the petri dish plate.
8. This step is performed to remove erythrocytes and free Leydig cells.
9. The alteration of osmolality caused by glycine solution (hyperosmotic) will lyse some testicular cell types, particularly those outside the blood-testis barrier (e.g. spermatogonia). It also makes it easier to remove cells from the basement membranes during enzymatic dissociation. EDTA is a chelating agent, i.e., it has the ability to binds to calcium and magnesium. The trypsin inhibitors protect the cells against proteolytic degradation.

10. Collagenase is essential for degrading the extracellular matrix. It plays a crucial role in the dissociation of tissues, thus contributing to the yield and viability of the cells.

11. The culture medium must be previously placed in a water bath at a temperature between 33-37 ºC.

12. Be careful with overdigestion.

13. The solution can be divided into 4 tubes of 15 ml to facilitate centrifugation.

14. Wash with a medium-soaked glass Pasteur pipette to release germ cells from agglomerates.

15. Every two days, the cells should be observed under the microscope to observe the status of the cultures and confluence. The culture medium should also be observed, for if it is a medium containing a pH indicator, such as Phenol red, it will change color when it is necessary to replace it.

16. This step is essential, not only to assess confluence but also to detect any contamination in the cell culture.

17. The washes should be done gently with care. PBS should not be directly added to the bottom of the flasks, where the cells adhere, so that they do not come loose. The flask should be shaken gently. If the cells are released during the washes, you can replace PBS with PBS supplemented with calcium chloride (0.9 mM) and magnesium chloride (0.49 mM).

References

1. Welsh, M.J. and J.P. Wiebe, 1975, Rat Sertoli Cells: A Rapid Method for Obtaining Viable Cells. Endocrinology, 96(3): p. 618-624.
2. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and Cellular Biochemistry, 408(1-2): p. 47-54.
3. Jesus, T.T., et al., 2014, Aquaporin-9 is expressed in rat Sertoli cells and interacts with the cystic fibrosis transmembrane conductance regulator. IUBMB Life, 66(9): p. 639-644.
4. Mather, J.P. and P.E. Roberts, Introduction to cell and tissue culture: theory and technique. 1998, California: Springer Science & Business Media.
5. Dutta, S. and P. Sengupta, 2016, Men and mice: relating their ages. Life Sciences, 152: p. 244-248.
6. Steinberger, A., et al., 1975, Isolation and culture of FSH responsive Sertoli cells. Endocrine Research Communications, 2(3): p. 261-272.
7. Orth, J.M., 1982, Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anatomical Record, 203(4): p. 485-492.
8. Majumdar, S.S., et al., 1995, Isolation and culture of Sertoli cells from the testes of adult Siberian hamsters: analysis of proteins synthesized and secreted by Sertoli cells cultured

from hamsters raised in a long or a short photoperiod. Biology of Reproduction, 52(3): p. 658-666.

9. Nagano, T. and F. Suzuki, 1976, The postnatal development of the junctional complexes of the mouse Sertoli cells as revealed by freeze-fracture. Anatomical Record, 185(4): p. 403-417.
10. Hatier, R. and G. Grignon, 1980, Ultrastructural study of Sertoli cells in rat seminiferous tubules during intrauterine life and the postnatal period. Anatomy and Embryology, 160(1): p. 11-27.
11. Bergmann, M. and R. Dierichs, 1983, Postnatal formation of the blood-testis barrier in the rat with special reference to the initiation of meiosis. Anatomy and Embryology, 168(2): p. 269-275.
12. Meng, J., et al., 2005, Androgens regulate the permeability of the blood–testis barrier. Proceedings of the National Academy of Sciences of the United States of America, 102(46): p. 16696-16700.
13. Oliveira, P.F., et al., 2009, Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. The Journal of membrane biology, 227(1): p. 49-55.
14. Reinhardt, V., F. Mutiso, and A. Reinhardt, 1978, Social behaviour and social relationships between female and male prepubertal bovine calves (Bos indicus). Applied Animal Behaviour Science, 4(1): p. 43-54.
15. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.
16. Meneses, M., et al., 2016, Pioglitazone increases the glycolytic efficiency of human Sertoli cells with possible implications for spermatogenesis. International Journal of Biochemistry and Cell Biology, 79: p. 52-60.

Chapter 4

Evaluation of the purity of Sertoli cell primary cultures

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Evaluation of the purity of Sertoli cell primary cultures

Abstract

The purity of the primary cultures of Sertoli cells is a factor for the validation of the studies using this methodological approach. There is a probability of contamination of these cultures with other testicular cellular types, such as peritubular cells and germ cells (that represent a large percentage of the volume of the seminiferous tubules). For the evaluation of the purity of cultures, the immunocytochemistry technique (immunoperoxidase or immunofluorescence) is frequently used to label a protein marker specific for the Sertoli cells within the testicular environment. The expression of several protein markers can be used, with different particularities, being that vimentin is often used as a marker for Sertoli cells. Vimentin is expressed independently of the differentiation state of the cells, and is observed around the nuclei and in the cytoplasm of Sertoli cells.

Introduction

Sertoli cell cultures are obtained from samples of total testis or from small explants of testicular tissue (biopsies). These cultures are prepared taking advantage of enzymatic and mechanical processes for removal of interstitial and peritubular tissues and cells, such as germ cells and somatic cells of peritubular origin [1,2]. The germ cells constitute the more frequent contaminants in Sertoli cell primary cultures, representing sometimes more than 25% of the cultured cells even when cells are obtained from prepubertal mice [3]. If the cells are obtained from older animals, the percentage of cell contaminants increases. These contaminants generally are removed with the exposure to conditions to which the Sertoli cell cultures are more resistant [4,5].

Immunocytochemistry is a technique for detecting molecules of interest within cells by means of immunological and chemical reactions. This technique is highly specific and sensitive and can detect a large variety of antigens [6]. Usually, in this technique, the antigen is recognized by a specific primary antibody, to which the secondary antibody binds. Then a reagent that will bind to the secondary antibody is added, which contains an enzymatic portion that will convert a substrate (eg: DAB) into a colored product. The color will appear in the same location where the primary antibody recognized that antigen of interest [7]. Alternatively, the antigen of interest can be observed using secondary antibody linked to a fluorophore (eg: FITC) [1], which is detected in a fluorescence microscope.

Immunocytochemistry is used for evaluating the purity of Sertoli cell cultures as this is a crucial factor for the validation of studies involving this experimental approach. The purity of the Sertoli cell cultures can be assessed by the immunocytochemistry, through the detection of protein marker specific for the Sertoli cells within the testicular environment [8,9].

Vimentin is the common intermediate filament protein that supports desmosome function. It is formed by polymerization of 57 kDa vimentin monomers and is an essential component of the Sertoli cell cytoskeleton [10,11]. It is knowing that this protein has an important role in anchoring germ cells to the seminiferous epithelium with consequent importance for normal spermatogenesis [12]. This protein is regularly present in Sertoli cells independent of spermatogenesis phase or the state of Sertoli cells differentiation [13]. At the structural level, vimentin is observed around the nuclei, in cytoplasm along fibrillary material and at the ectoplasmic specializations, and also in the periphery of the Sertoli cell processes [10,13]. Hence, this filament protein is often used as a specific marker for Sertoli cells [4,7].

In this protocol two possible procedures are described for evaluating the purity of the primary cultures of Sertoli cells through the detection of a specific protein marker, vimentin. They involve the examination of cell cultures by microscopy techniques and their selection based on the presence of other testicular cell contaminants below 10 to 5%. Only under these conditions one can state to have a pure culture of Sertoli cells.

Material

All the solutions used in this procedure should be prepared with ultrapure water and stored at room temperature or kept at 4ºC (unless indicated otherwise).

Working solutions

1. Phosphate-buffered Saline Solution (PBS): 140 mM sodium chloride, 3 mM potassium chloride, 10 mM sodium phosphate dibasic and 1.8 mM potassium phosphate monobasic anhydrous. Weigh all the chemicals into a 1 L graduated cylinder and dissolve in 900 mL of water. If necessary, the pH of the solution should be adjusted to 7.4, using concentrated solutions of hydrochloric acid or/and sodium hydroxide. Adjust the volume to 1 L.

2. Phosphate-buffered Saline Solution with Tween 20 (PBS-T): Prepare PBS as referred above and add 2 mL Tween 20.

3. Fixing solution: PBS with 4% (w/v) paraformaldehyde, pH 7.4.

4. Antigen retrieval buffer: 10 mM Sodium citrate solution diluted in ultrapure water, pH 6.

5. Inhibition solution: Methanol with 3% (v/v) hydrogen peroxide.

6. Permeabilization solution: PBS with 0.1% (v/v) Triton X-100.

7. Blocking solution: PBS-T with 1% (w/v) BSA or PBS-T with 10% (v/v) serum from the species from which the secondary antibody was obtained.
8. Extravidin-peroxidase complex solution (2-2.5 mg/mL)
9. 3,3'-Diaminobenzidine (DAB) Substrate Solution
10. DAPI or Hoechst 33342 solution (10 mg/mL)
11. Hematoxylin solution (1 mg/mL) diluted in ethanol.
12. Primary antibody (anti-Vimentin) solution (1 mg/mL)
13. Fluorophore-conjugated secondary antibody or horseradish peroxidase (HRP)-conjugated secondary antibody solution.

Methods

Sertoli cell cultures purity can be revealed by immunocytochemistry by fluorescence (Protocol A) or by immunoperoxidase technique (Protocol B). Cultures can be examined by fluorescence microscopy or by phase contrast microscopy and can be used if the other cell contaminants are below 5-10%.

- **Preparing the slide (Protocol A and B)**

1. Sterilize glass coverslips by dipping them in 90% ethanol and carefully drying in the laminar flow.
2. Place each coverslip in sterile 6-well culture plates.
3. Add about 1 mL of cell suspension over each coverslip in the plate.
4. Grow cells on glass coverslips at 33 ºC in an incubator (5% CO_2) during 96 hours.
5. Aspirate the culture medium from each well and gently rinse the cells 3 times in PBS at room temperature (see Note 1).

- **Fixation (Protocol A and B)**

1. Incubate the cells with fixing solution for 10 minutes at room temperature.
2. Wash the cells 3 times with ice cold PBS (see Note 2).

- **Antigen retrieval (see Note 3) (Protocol A and B)**

1. Heat the antigen retrieval buffer to 98 ºC (see Note 4).
2. With the help of one tweezer, dip the coverslips in the buffer, being careful to know which side of the coverslip has the cells.
3. Heat the coverslips at 98°C for 10 minutes.

4. Remove the coverslips from the antigen retrieval buffer and put them in the 6-well culture plates with the cells facing up.
5. Wash the coverslips with cells 3 times in PBS, 5 minutes each wash.

- **Permeabilization (Protocol A and B)**

1. Incubate the coverslips with the cells in permeabilization solution for 15 minutes at room temperature (see Note 5).
2. Wash the coverslips with cells 3 times in PBS, 5 minutes each wash

- **Inhibition of endogenous peroxidases (Protocol B)**

1. Incubate the cells with inhibition solution for 10 minutes at room temperature.
2. Wash the coverslips with cells 2 times in PBS-T, 5 minutes each wash.

- **Blocking and immunostaining (Protocol A)**

1. Incubate the coverslips with the blocking solution for 1 hour at room temperature to block unspecific binding of the antibodies.
2. Wash the coverslips with cells 3 times in PBS-T, 5 minutes each wash.
3. Incubate the coverslips with primary antibody (anti-Vimentin) diluted in blocking solution, with an appropriate dilution, overnight at 4 ºC in a humidified chamber (see Note 6). Use a humidity chamber for all incubations to prevent evaporation. The final volume of the antibody solution should be sufficient to cover each coverslip (see Note 7).
4. Wash the coverslips with cells 3 times in PBS-T, 5 minutes each wash.
5. Incubate the coverslips with the fluorophore-conjugated secondary antibody diluted in blocking solution with a respective dilution 1 hour at room temperature in the dark (see Note 8).
6. Wash the coverslips with cells 3 times in PBS-T, 5 minutes each wash.
7. Incubate the coverslips with DAPI or 3 µg/mL Hoechst 33342 stain for 10 minutes (see Note 9).
8. Wash the coverslips with cells 3 times in PBS-T, 5 minutes each wash.

- **Blocking and immunostaining (Protocol B)**

1. Incubate the coverslips with blocking solution for 1 hour at room temperature to block unspecific binding of the antibodies.
2. Wash the coverslips with cells 2 times in PBS-T, 5 minutes each wash.

3. Incubate cells with primary antibody (anti-Vimentin), diluted in blocking solution with an appropriate dilution, overnight at 4 ºC in a humidified chamber (see Note 6). Use a humidity chamber for all incubations to prevent evaporation. The final volume of the antibody solution should be sufficient to cover each coverslip (see Note 7).

4. Wash the coverslips with cells 3 times in PBS-T, 5 minutes each wash.

5. Incubate coverslips with HRP secondary antibody solution, diluted in blocking solution with an appropriate dilution, during 1 hour at room temperature.

6. Wash the coverslips with cells 3 times in PBS-T, 5 minutes each wash.

7. Incubate the coverslips with the extravidin-peroxidase complex diluted in blocking solution for 30 minutes (see dilution and incubation time recommended by the manufacturer).

8. Wash the coverslips 2 times in PBS-T, 5 minutes each wash.

9. Add the DAB Substrate Solution (see Note 10) to the coverslips and incubate until the desired development is achieved (a brownish color appears). Typical incubations are 5 to 15 minutes long (see Note 11).

10. Stop the reaction by washing the coverslips with water for 2 minutes.

11. Immerse 4 times in hematoxylin solution for 10 seconds to stain cell nuclei (optional step) (see Note 12).

12. Wash the coverslips under running water.

- **Mounting of the coverslips and visualization (Protocol A and B)**

1. Mount the coverslips in a slide with a drop of mounting medium (see Note 13).

2. Seal the coverslips on the slides with nail polish to prevent drying and movement.

3. Visualize the cells using a fluorescence microscope with the appropriate filters (Protocol A) or optical microscope (Protocol B) (Figure 4.1). Store the coverslips in the dark at -20 ºC.

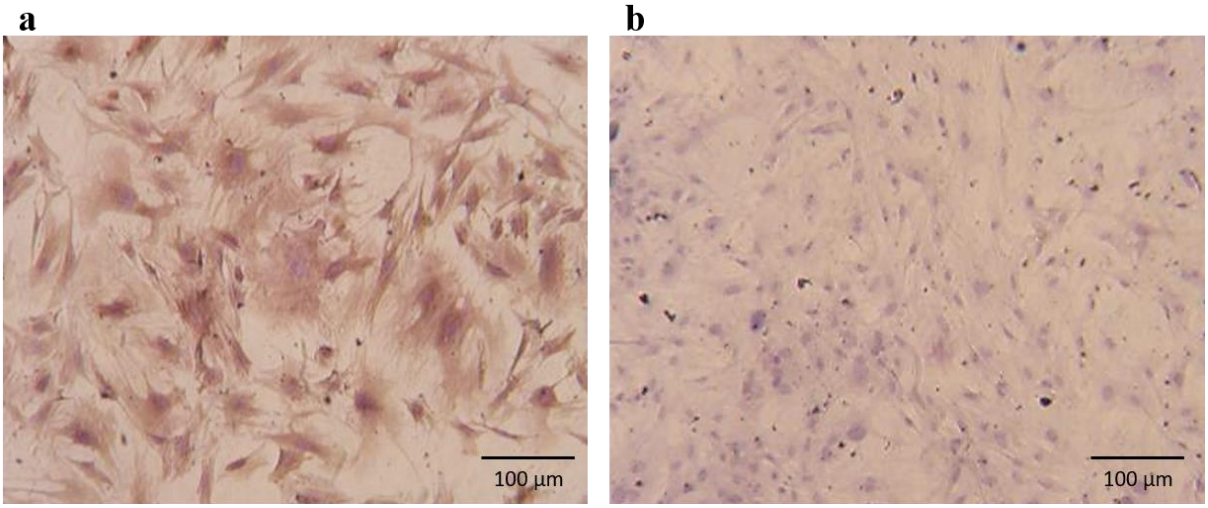


Figure 4.1 Immunocytochemistry of a culture of Sertoli cells. **(a)** Marking of vimentin in Sertoli cells observed under the optical microscope. **(b)** Negative control, without adding any antibodies and nuclei stained with hematoxylin.

Notes

1. PBS must be preheated to prevent the releasing of the cells from the coverslip.
2. The coverslips containing the cells can be stored in PBS with 0.02% sodium azide in PBS at 4°C for a few days.
3. This step is optional. Antigen retrieval is a methodological approach that increases the availability of epitopes that can be bound by the specific primary antibody. Some antibodies bind better when cells are heated in antigen retrieval buffer. Check the product data sheet recommendations.
4. The heating of the solution should be done in a water bath to maintain the temperature constant during incubation. In this step, one must be careful with the temperature, because when heating methods such as microwave are utilized, this can result in unbalanced epitope retrievals due to a creation of cold and hot spots. A temperature too high may also cause the cells dissociation from the coverslip.
5. Triton X-100 is the detergent more frequently used for permeabilization. However, this method tends to destroy some of the membranes, and for that reason it is not appropriated for membrane associated antigens. Digitonin or saponin are also agents that improve the penetration of the antibody, and which can replace Triton X-100.
6. If the antibodies used have not been previously assayed for these cells, specific control experiments should be performed to evaluate if the antibodies react with all isotypes, which are:
- Perform the entire protocol without adding any antibodies. Any fluorescence observed is due to the autofluorescence of the sample.
- Perform the entire protocol without adding the primary antibody. Any observed fluorescence is due to non-specific binding of the secondary antibody.
7. If possible use a hydrophobic pen, the antibody will not spread as easily over the coverslip.
8. After this step, all procedures must be performed in the dark, so that no fluorescence is lost, and at room temperature.
9. DAPI or Hoechst 33342 will stain the nuclei of the cells.
10. DAB substrate can be purchased in powder or liquid form. If bought in powder dissolve in 50mM Tris, pH 7.2 at 1 mg/mL. The manufacturer's instructions should be followed to make the solution.
11. DAB reaction produces brown bands or spots at sites of reaction with HRP-conjugated antibodies.
12. If the staining is too strong, the immersion times in hematoxylin can be reduced.

13. Take out the coverslips with tweezers and place them on the mounting medium, with the cell-side face down.

References

1. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.
2. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and Cellular Biochemistry, 408(1-2): p. 47-54.
3. Palombi, F., et al., 1979, Morphological characteristics of male germ cells of rats in contact with Sertoli cells in vitro. Journal of Reproduction and Fertility, 57(2): p. 325-330.
4. Rato, L., et al., 2012, Metabolic modulation induced by oestradiol and DHT in immature rat Sertoli cells cultured in vitro. Bioscience Reports, 32(1): p. 61-69.
5. Orth, J.M., 1982, Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anatomical Record, 203(4): p. 485-492.
6. Polak, J.M. and S. Van Noorden, Immunocytochemistry: Practical Applications in Pathology and Biology. 2014, Bristol: Butterworth-Heinemann.
7. Alam, M.S. and M. Kurohmaru, 2014, Disruption of Sertoli cell vimentin filaments in prepubertal rats: An acute effect of butylparaben in vivo and in vitro. Acta Histochemica, 116(5): p. 682-687.
8. Griswold, M.D., Sertoli cell biology. Second ed. 2014, Amsterdam: Academic Press.
9. Yakimchuk, K., 2013, Cell Markers. Materials and Methods, 3: p. 183.
10. Mruk, D.D., M. Bonanomi, and B. Silvestrini, 2016, Lonidamine-ethyl ester-mediated remodelling of the Sertoli cell cytoskeleton induces phosphorylation of plakoglobin and promotes its interaction with α-catenin at the blood–testis barrier. Reproduction, fertility, and development, 29(5): p. 998-1011.
11. Franke, W.W., et al. Differentiation-related patterns of expression of proteins of intermediate-size filaments in tissues and cultured cells. in Cold Spring Harbor Symposia on Quantitative Biology. 1982. Cold Spring Harbor Laboratory Press, 46: 431-453.
12. Amlani, S. and A. Vogl, 1988, Changes in the distribution of microtubules and intermediate filaments in mammalian Sertoli cells during spermatogenesis. Anatomical Record, 220(2): p. 143-160.
13. Aumüller, G., et al., 1988, Distribution of vimentin-type intermediate filaments in Sertoli cells of the human testis, normal and pathologic. Anatomy and Embryology, 178(2): p. 129-136.

Chapter 5

Carbonic anhydrases are involved in mitochondrial biogenesis and control the production of lactate by human Sertoli cells

This chapter was adapted from the submitted work:

***Raquel L. Bernardino**, et. al., Pedro F. Oliveira. Carbonic anhydrases are involved in mitochondrial biogenesis and control the production of lactate by human Sertoli cells. Submitted*

Carbonic anhydrases are involved in mitochondrial biogenesis and control the production of lactate by human Sertoli cells

Abstract

The process that allows cells to control their pH and bicarbonate levels is essential for ionic and metabolic equilibrium. Carbonic anhydrases (CAs) catalyse the conversion of CO_2 to HCO_3^- and H^+ thus being essential for those processes. Herein, we studied the involvement of CAs (by inhibition with acetazolamide - ACT and SLC-0111) in the metabolism, mitochondrial potential, mitochondrial biogenesis and lipid metabolism of human Sertoli cells (hSCs), obtained from biopsies from men with conserved spermatogenesis. We were able to identify three isoforms of CAs, one mitochondrial isoform (CA VB) and two cell membrane-bound isoforms (CA IX and CA XII) in hSCs. When assessing the expression of markers for mitochondrial biogenesis, we observed a decrease in *HIF-1α*, *SIRT1*, *PGC1α* and *NRF-1* mRNAs after all CAs were inhibited. This was followed by an increased production of lactate and alanine in the same condition. In addition, consumption of glucose was maintained after inhibition of all CAs in hSCs. These results indicate a reduced conversion of pyruvate to acetyl-coA, possibly due to diminished decreased mitochondrial function, caused by CAs inhibition in hSCs. Inhibition of CAs also caused alterations in lipid metabolism since we detected an increased abundance of *HSL* in hSCs. Our results suggest that CAs are essential for the expression of genes involved in mitochondrial biogenesis, glucose and lipid metabolism of hSCs. This is the first report showing that CAs can play a key role in spermatogenesis by controlling lactate production and modulating the expression of genes associated with mitochondrial biogenesis in hSCs.

Keywords: Acetazolamide, Bicarbonate, metabolism, mitochondria biogenesis, Sertoli cells.

Introduction

Most biochemical processes involved in the control of cellular function, growth and development depend on the establishment of a proper *millieu*. Biological molecules, like proteins, phosphatases and amino acids are weak bases and weak acids that can bind or release protons (H^+). To control intracellular and extracellular milieu, cells need a high pH buffering power [1]. Among these acid/base loads, cellular respiration is the responsible for producing H^+-yielding CO_2 or lactic acid. The CO_2 produced in the cytosol can be associated

with H_2O to form H_2CO_3, most of which slowly dissociates to form HCO_3^- and H^+ [2]. Carbonic anhydrases (CAs) are a group of enzymes presents in the most cells, this enzyme catalyzes the reversible hydration of CO_2 to H_2CO_3 and thus accelerates the process of cells acidification [3].

In living organisms, CAs are widely distributed, differing in tissue localization and subcellular distribution. CAs are a group of isozymes that differ in kinetic properties, inhibitor sensitivity and selectivity. To date, 15 isoforms have been described in humans that differ primarily in their subcellular localization. CA IV and CA XIV are cell membrane-bound isoforms, CA IX, XII, and XIV are transmembrane isoforms while CA I, CA II, CA III, CA VII, CA VIII, CA X, CA XI and CA X III are cytosolic isoforms [2]. CA VI is secreted and CA V (A and B) is the only mitochondrial isoform [4, 5]. Acetazolamide (ACT) was the first compound non-mercurial diuretic clinically used as a general inhibitor of CAs [6]. Indeed, CAs inhibitors have been clinically used for the treatment of several pathologies such as glaucoma, epilepsy, ulcers, neurological disorders, cancer, among others. Actually, more specific inhibitors have been developed because of the large number of isoforms of CAs and their involvement with clinical pathologies [6, 7].

Acid-base homeostasis in the male reproductive tract is very important for reproduction, by influencing a series of processes involved in spermatogenesis. Sertoli cells (SCs) play a key role in male fertility by the control and support of spermatogenesis. Those include the establishment of the blood-testis barrier, nutrition, physical support and secretion of all factors essential for development of germ cell line, the modulation of the apoptosis and phagocytosis of spermatogenic cells and production of the seminiferous tubular fluid (STF) [8]. In the male reproductive tract, the composition of STF is distinct from the blood and other body fluids and this is essentially due to SCs [9-11].

So far little the role of CAs in male reproductive tract and particularly in SCs remains obscure. Nevertheless, it is of great importance to understand the role of CAs for physiological processes, both in normal and pathological states, including in the male reproductive tract. Thus, we aimed to evaluate the effects of general and selective inhibition of CAs in the physiology and metabolism of human SCs (hSCs).

Material and Methods

Chemicals

Foetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany), Tween 20 from Applichem (Darmstadt, Germany) and dried milk from Regilait (Saint-Martin-Belle-Roche, France). Mammalian Protein Extraction Reagent and BCA Protein Assay Kit were purchased from Thermo Scientific (Whalthan, MA, USA). Dulbecco's modified eagle medium, Ham's nutrient mixture F12 (DMEM: Ham's F12), bovine serum albumin (BSA), Trypsin-EDTA, Insulin-Transferrin-Selenium supplement (ITS supplement) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise.

Patient's selection, ethical issues and testicle tissue preparation

Testicular biopsies were obtained from patients seeking for fertility treatment for recovery of male gametes. In the present study only cells left in the tissue culture plates after patients' treatment were used. hSCs were isolated from six testicular biopsies selected from patients with conserved spermatogenesis and seeking for treatment due to anejaculation (psychological, vascular, neurologic), vasectomy or traumatic section of the vas deferens. Each testicle biopsy was collected in sperm preparation medium (SPM-Hepes buffer; Medicult, Florence, Italy) and kept at 33°C with 6% CO_2 in air until use. The experiments were undertaken with the understanding and written consent of each subject. The study methodologies conformed to the standards set by the Declaration of Helsinki, and were approved by the local ethics committee.

Primary culture of hSCs

Primary culture of hSCs were obtained by following the protocol described by Bernardino et al. [50]. In brief, human testicular biopsies were washed twice in ice-cold Hank's balanced salts solution (HBSS) (in g/L: potassium chloride 0.4, potassium phosphate monobasic anhydrous 0.06, sodium chloride 8, sodium phosphate dibasic 0.045, D-glucose 1, sodium bicarbonate 0.35 and pH 7.4) by centrifuging at 500x*g,* room temperature during 5 minutes. The resulting cellular pellet was suspended in Sertoli culture medium, which consisted of a 1:1 mixture of DMEM: Ham F12, supplemented with 15 mM HEPES, 50 U/mL penicillin, 50 mg/mL streptomycin sulphate, 0,5 mg/mL fungizone, 50 µg/mL gentamicin and 10% inactivated FBS. In order to disaggregate large SC clusters, the cellular suspension was forced though a 20G needle. For cell culture, the concentration was adjusted to 5000 clusters/mL in Sertoli culture medium, plated on 75 cm^2 culture flasks (Cell+; Sarstedt, Nümbrecht, Germany), and incubated at 33ºC in an atmosphere of 6% CO_2: 94% O_2.

Experimental groups

hSCs were grown until reaching a confluence of 90%–95%. Then the culture medium was replaced by serum and phenol-red free medium (DMEM: F12 supplemented) with Insulin-transferrin-selenium supplement (ITS; in mg/L: insulin 10, transferrin 5.5, selenium 0.0067), pH 7.4. The cells grew in a medium with physiological concentrations of testosterone (5 µM) and estradiol (1 nM). In order to evaluate the effect of CA the cells are divided in three different groups, group control without any treatment, group treated with a general inhibitor for CAs, the ACT (10 µM), and the group treated with the specific inhibitor SLC-0111 (1 µM). SLC-0-011 is a potent CA inhibitor for CA IX and CA XII, against two other physiologically relevant CA isoforms (CA I and CA II) [51, 52]. Control (CTR) group was treated with same amount of solvent (ethanol) used in ACT and SLC-0111 groups (<0.025% v/v). Our group and others tested the ethanol dose used, and the results showed that it does not alter the conditions of cultured SCs [53]. Treatments were performed during 24 hours in an atmosphere of 6% CO_2, 94% O_2 at 33ºC.

Intracellular pH measurement

hSCs were seeded in black polystyrene 96-well plate, until reaching a confluence of 90%–95%. The cells were then divided in CTR and ACT groups, as previously described. Intracellular pH (pHi) was measured with a fluorescent probe using the method described by Bernardino and collaborators [20]. Briefly, hSCs were loaded with a fluorescent probe during 15 min at 37ºC with Control Solution (in mM: NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 1.8; KH_2PO4 1.8; $NaHCO_3$ 25; Glucose 14; HEPES 25; pH 7.4) containing 10 µM of BCECF–AM ester and 0.2% of Pluronic F-127. The fluorescence intensities, excited at 490 and 440 nm (fixed emission at 535 nm), were continuously measured with a fluorescence reader plate (SpetraMax Gemini EM; Molecular Devices), while the cells were perfused with different solutions. Background fluorescence was determined at the end of each experiment. The background fluorescence intensity was measured for each wavelength. The fluorescence ratios (F490/F440) were calculated after subtracting the background fluorescence intensities for each measurement at each wavelength. Probe calibration was performed at the end of each protocol calibration, using solutions of known pH (5.5, 7.4, 8 and 9.0) to which nigericin (10 mM) was added. To convert the measured fluorescence ratio (F490/F440) to pHi values, the equation described by Oliveira and collaborators [54] was used. The pK of BCECF had an average value of 7.01 ± 0.21 in our experimental conditions. In order to evaluate the impact of ACT on intracellular H^+ dynamics, changes in pHi were determined in hSCs from the different experimental groups, following the addition of ATP (2 mM).

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were acquired as previously described [17]. Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) to quantify the following metabolites present in solution (multiplet, ppm): lactate (doublet, 1.33); alanine (doublet, 1.47), acetate (singlet, 1.90), pyruvate (singlet, 2.36) and H1-a-glucose (doublet, 5.22). The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc., Fremont, CA, USA).

Lactate dehydrogenase activity assay

Lactate dehydrogenase (LDH) activity was determined using a commercial assay kit (Thermo Scientific, Waltham, MA) and following the manufacturer's instructions. In brief, LDH activity was calculated by measuring the shift on the absorbance (490 nm) of protein samples that resulted from the conversion of a tetrazolium salt into a red formazan product. The amount of formazan formed is directly proportional to the activity of LDH on the samples. LDH activities were calculated as units per milligram of protein using the molar extinction factor and final expressed as fold variation to the control group.

Western blot

Total protein fraction was extracted from hSCs using Mammalian Protein Extraction Reagent (supplemented with 1% protease inhibitor cocktail and 100 mM sodium orthovanadate) following the manufacturer's instructions. Protein concentration was determined by Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Protein samples (100 µg) were mixed with sample buffer (1.5% Tris, 20% glycerol, 4.1% SDS, 2% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8), denatured for 15 min at 37 °C. Proteins were fractionated in 15% polyacrylamide gels and electrophoresis was carried out for 90 min. The proteins were transferred from gels to previously activated polyvinylidene difluoride membranes in a Mini Trans-BlotR cell (Bio-Rad, Hemel Hempstead,UK) during 75 min and then blocked for 3 hours in a 5% non-fat milk solution at room temperature. The membranes were then incubated overnight at 4 °C with MitoProfileR Total OXPHOS WB Antibody Cocktail (1:1000, ab110413, Abcam, Cambridge, MA, USA). Membranes were reacted with ECL™ and read with the Bio-Rad QuemiDoc XR (Bio-Rad, Hemel Hempstead, UK). Quantity One Software (Bio-Rad, Hemel Hempstead, UK) was used to obtain band densities following standard procedures. The band density was divided by the total loaded protein (obtained by Ponceau S staining) and then normalized with the control group value.

Determination of the mitochondrial membrane potential

The mitochondrial membrane potential was determined using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Life Technologies, Gaithersburg, MD, USA). After treatment with CA inhibitors (ACT and SLC-0111), JC-1 was added to the media in a final concentration of 2 μM and the cells were incubated at 37 °C for 30 min. The media with JC-1 were then replaced by ITS media. Fluorescence of each sample was read at excitation wavelength of 485 nm and 535 nm and emission wavelength of 530 nm and 590 nm using Cytation™ 3 (Biotek Instruments Inc., Winooski, VT). In healthy mitochondria, a high concentration of JC-1 forms aggregates that yield red fluorescence at 590 nm. In unhealthy mitochondria, JC-1 exists as a monomer at low concentration emitting green fluorescence at 530 nm. Results in fluorescence intensity were expressed as the ratio of 590 nm to 530 nm emission.

Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qPCR)

Extraction of RNA from hSCs was performed using the E.Z.N.A. tRNA Kit (Omega Bio-Tek, Norcross, USA) as indicated by the manufacturer. RNA concentrations were determined by Nanodrop 2000 Spectrophotometers (Thermo Fisher Scientific, California, USA). RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase (NZYtech, Lisboa, Portugal). The resultant complementary DNA (cDNA) was used with exon-exon spanning primer sets designed to amplify cDNA fragments described in Table 5.1.

Conventional reverse transcriptase PCR (RT-PCR) was performed to identify the mRNA expression of *CA VB*, *CA IX* and *CA XII*. Quantitative Real-time PCR (qPCR) was performed to evaluate the mRNA abundance of *CA VB*, *CA IX*, *CA XII*, *Hypoxia-inducible factor 1-alpha (HiF1-α)*, *Nuclear respiratory factor-1 (NRF-1)*, *peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), Sirtuin 1 (SIRT1)*, *hormone sensitive lipase (HSL)* and *β2-microglobulin*. Specific primers were designed for amplification of the target and housekeeping transcripts. *β2-microglobulin* transcript levels were used to normalize gene expression levels. Fold variation of gene expression levels was calculated following the mathematical model proposed by Pfaffl [18], using the formula $2^{-\Delta\Delta Ct}$.

A qPCR method was also used to determine the mtDNA copy number. The DNA specimens were prepared using the SurePre RNA/DNA/Protein Purification Kit (Thermo Fisher Scientific, California, USA). qPCR was performed to evaluate the DNA abundance of mitochondrial *NADH dehydrogenase 1 (MT-ND1)*, that was normalized with *β2-microglobulin* (single copy gene). The average of all three measurements was calculated.

Table 5.1 Oligonucleotides and cycling conditions for PCR amplification of Carbonic anhydrase VB (CA VB), *Carbonic anhydrase IX* (*CA IX*), *Carbonic anhydrase XII* (*CA XII*), *Hypoxia-inducible factor 1-alpha* (*HiF1-α*), *Nuclear respiratory factor-1* (*NRF-1*), *Sirtuin 1* (*SIRT1*), *peroxisome proliferator-activated receptor γ coactivator 1α* (*PGC1α*), *hormone sensitive lipase* (*HSL*), *mitochondrial NADH dehydrogenase 1* (*MT-ND1*) and β-2-microglobulin. Ann. Tº: Annealing temperature; C: Number of cycles.

Gene	Sequence 5'- 3'	Ann. Tº	C
CA VB (NM_007220.3)	**FWD:** AGAGACGCTCAGATTATTAAGTTCC **RVS:** GTCCCTCCACCGAATGTTGA	60ºC	35
CA IX (NM_001216.2)	**FWD:** TAGCCCTGGTTTTTGGCCTC **RVS:** GCAGGACAGGACAGTTACCG	58ºC	35
CA XII (NM_001293642.1)	**FWD:** TGGACTTATTTTGTGAAGCTGAACC **RVS:** CGAATGCTTCCTGGCCTTTG	58ºC	35
HIF1-α (NM_001530.3)	**FWD:** GATCACCCTCTTCGTCGCT **RVS:** CCCCTCGTGAGACTAGAGAGA	58ºC	40
NRF-1 (NM_005011.4)	**FWD:** CAGCCGCTCTGAGAACTTCA **RVS:** GGCCGTTTCCGTTTCTTTCC	58ºC	40
SIRT1 (NM_001314049.1)	**FWD:** ACAGGTTGCGGGAATCCAAA **RVS:** GTTCATCAGCTGGGCACCTA	60ºC	40
PGC1α (NM_013261.3)	**FWD:** TGGTTGCCTGCATGAGTGT **RVS:** CACCACTTGAGTCCACCCAG	56ºC	40
HSL (NM_005357.3)	**FWD:** CCCACCAAAGAAAGAGGCAC **RVS:** GCTCTAGCGGGGTTATAGGC	58ºC	35
MT-ND1 (NC_012920.1)	**FWD:** CGATTCCGCTACGACCAACT **RVS:** AGGTTTGAGGGGGAATGCTG	58ºC	40
β-2-Microglobulin (NM_004048.2)	**FWD:** AGATGAGTATGCCTGCCGTG **RVS:** TCATCCAATCCAAATGCGGC	58ºC	35
β-2-Microglobulin (NC_000015.10)	**FWD:** GAGGCTATCCAGCGTGAGTC **RVS:** GACGCTTATCGACGCCCTAA	58ºC	35

Staining of intracytoplasmic lipids

hSCs were seeded in sterile microscope coverslips in 24-well plates. After reaching about 70% confluence, cells were treated with ACT medium or with control medium during 24 hours. At the end of treatment, hSCs were washed with phosphate buffered saline (PBS) and fixed for 5 min with 10% neutral buffered formalin. Cells were washed with distilled water and 85%

propylene glycol (Merck Millipore, USA) was added, for 2 min and changed 2 times. Then, Oil Red O (0.5% in propylene glycol) was spread evenly over the cells and left for 30 min at room temperature. Oil Red O was removed and cells washed with 85% propylene glycol, for 1 min and repeated 2 times. After a washing step with distilled water, cells were stained with hematoxylin (Merk Millipore, USA) for 30 s. Hematoxylin is a dye that stains the cell nucleus. Finally, cells were washed one last time with distilled water and mounted with Aquatex® (Merck Millipore, USA). hSCs with staining lipid droplets were observed and the images were recorded using an Olympus DX50 inverted light microscope (Tokyo, Japan) equipped with an Olympus DP21 digital camera.

Statistical Analysis

Experimental data are shown as mean ± standard error of mean (SEM) (n=6 for each condition, done in triplicate). Statistical analysis was performed using a one-way ANOVA in GraphPad Prism 6 (GraphPad Software, San Diego, USA). $P<0.05$ was considered significant.

Results

Carbonic anhydrase VB, IX and XII isoforms are expressed in hSCs

To date, the studies on the role and location of the various isoforms of CA in the human testis and particularly in SCs are scarce. In this study we performed a screen for CAs in hSCs. We were able to identify by PCR the expression of three isoforms of CA in hSCs. We identify the mRNA of *CA VB*, *IX* and *XII* (Figure 5.1a). The positive control for CA IX and XII was RT4 cell line (obtained from urinary bladder carcinoma) [12, 13], and for CA VB was used human liver [14]. The relative abundance of the three isoforms was quantified by qPCR using *β2-microglobulin* as housekeeping, with the primer efficiency being set to 90% - 110%. In hSCs, the relative abundance of *CA IX* was 0.031 ± 0.015, *CA XII* of 0.005 ± 0.003 and *CA VB* was 0.078 ± 0.042 arbitrary units (Figure 5.1b).

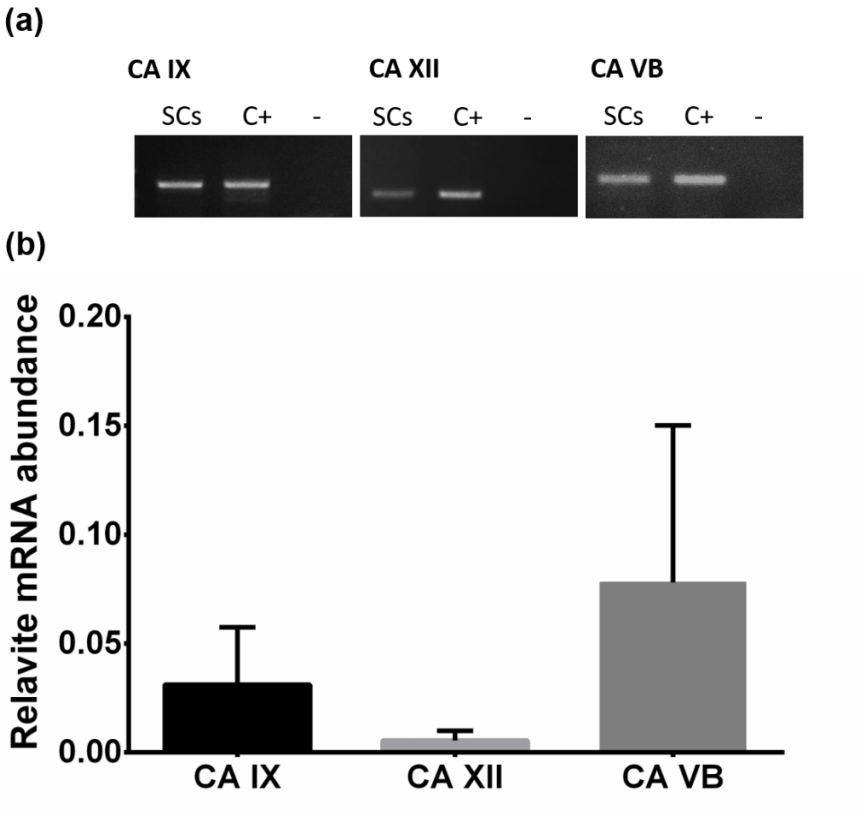


Figure 5.1 Identification and quantification of mRNA abundance of carbonic anhydrase IX (CA IX), carbonic anhydrase XII (CA XII) and carbonic anhydrase VB (CA VB) in human Sertoli cells (hSCs). **a)** Illustrative PCR experiment. **b)** Quantification of mRNA abundance of CA IX, CA XII and CA VB normalized with β-2-microglobulin. Results are expressed as mean ± standard error of mean (SEM) (n = 6).

Carbonic anhydrase is not involved in the control of hSCs intracellular pH

Our results show hSCs in both, CTR group and the group treated with ACT, exhibited a similar pHi. Under control conditions pHi value was 6.65 ± 0.02 and when exposed to ACT and thus, CAs were inhibited, this value was maintained to 6.65 ± 0.01 (Figure 5.2a). hSCs possess functional purinoreceptors that are involved in the regulation of ion homeodynamics and particularly H^+ dynamics. Hence, we evaluated the influence of ATP on intracellular H^+ concentration in cells treated with ACT and in cells from the CTR group, using the same fluorescent probe BCECF-AM. Exposure of hSCs to ATP induced a pHi shift of 0.08 ± 0.03 that was not significantly different from the shift detected in hSCs treated with ACT, where the pHi shift was 0.06 ± 0.02 (Figure 5.2b).

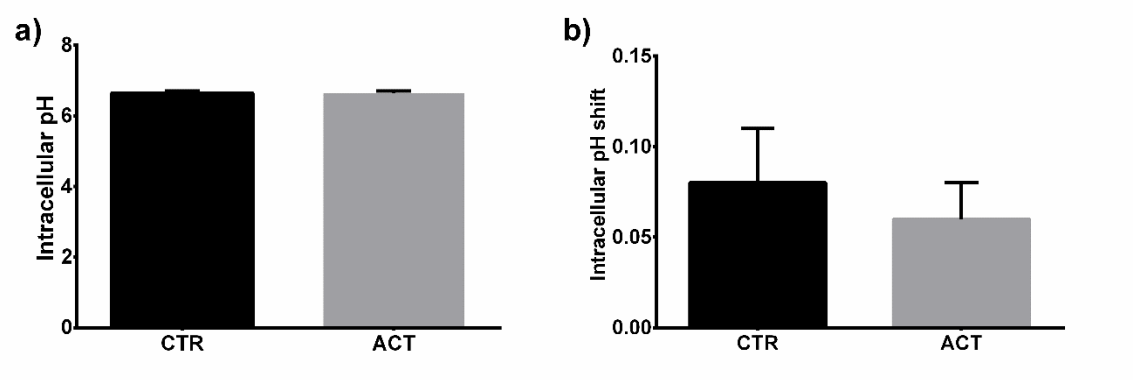


Figure 5.2 Effect of acetazolamide (ACT) on human Sertoli cells (hSCs) intracellular pH. **a)** The intracellular pHi in the control group (CTR) and ACT-treated hSCs is shown. **b)** The intracellular pH shift in both groups (control and ACT group) after stimulation with ATP is shown. Results are expressed as mean ± standard error of mean (SEM) (n = 6 for each condition).

Carbonic anhydrase modulates lactate and alanine production by hSCs

Pyruvate formed through glycolysis or attained from extracellular media can be converted into lactate and alanine. Our data showed no significant alterations in pyruvate production by hSCs treated with ACT (0.32 ± 0.09 pmol/cell), when compared with cells from the CTR group (0.27 ± 0.14 pmol/cell) (Figure 5.3a). However, hSCs treated with ACT produced higher quantities of lactate (11.20 ± 0.49 pmol/cell) and alanine (0.56 ± 0.07 pmol/cell) compared with cells from the CTR group which produced 5.5 ± 1.94 pmol/cell and 0.22 ± 0.12 pmol/cell of lactate and alanine respectively (Figure 5.3b and c). No alteration was observed in glucose consumption when comparing hSCs exposed to ACT (226.83 ± 21.92 pmol/cell) and non-exposed cells (196.2 ± 42.76 pmol/cell) (Figure 5.3d). No differences were also detected in the acetate production by hSCs where CAs were inhibited (19.5 ± 0.060 pmol/cell) when comparing with those cells where CAs remain active (0.174± 0.070 pmol/cell) (Figure 5.3e). In addition, LDH activity in hSCs treated with ACT showed no alteration when comparing to the one detected in cells from the CTR group (1.02 ± 0.15-fold variation to control) (Figure 5.3f).

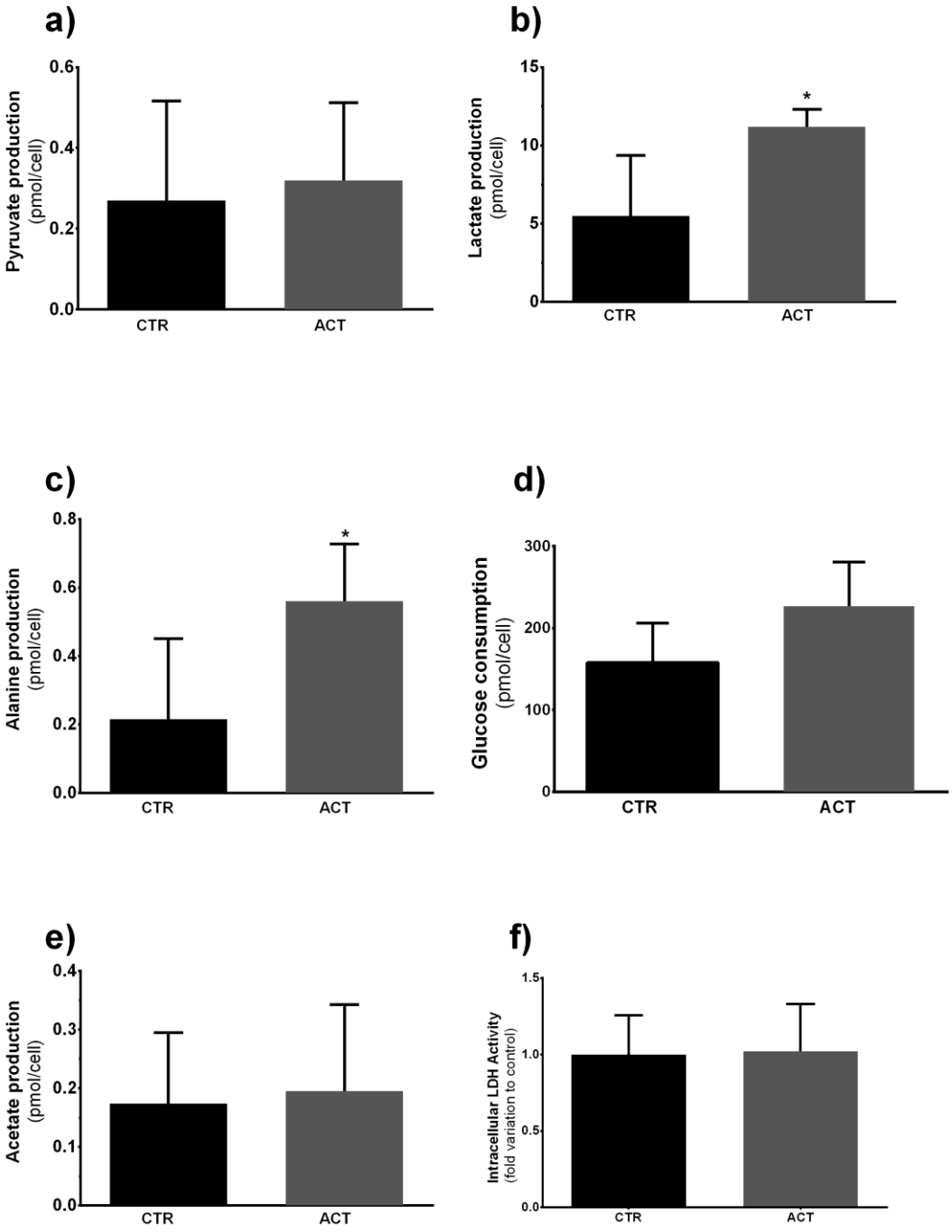


Figure 5.3 Effect of acetazolamide (ACT) in pyruvate production **(a)**, lactate production **(b)**, alanine production **(c)**, glucose consumption **(d)** acetate production **(e)** and intracellular LDH activity **(f)** in human Sertoli cells (hSCs). Results are expressed as mean ± standard error of mean (SEM) (n = 6 for each condition). *Significantly different relative to control ($p<0.05$).

Carbonic anhydrase inhibition causes a decrease in the expression of genes related with mitochondrial biogenesis

We evaluated the expression of key genes involved in mitochondria dynamics to assess the role of CA in hSCs bioenergetics. The inhibition of CA in hSCs alters the expression of genes involved in mitochondrial biogenesis and dynamics by modulating *SIRT1*, *PGC1α*, *NRF-1* and *HIF1-α* expression levels. *SIRT1* is involved in cellular energy metabolism and its relative abundance is decreased in ACT-treated cells (0.03 ± 0.005 arbitrary units) in relation with the expression detected in cells from the CTR group (1.062 ± 0.04 arbitrary units). However, when hSCs are exposed to SLC-0111 we did not observe any alteration in *SIRT1* abundance (0.62 ± 0.37 arbitrary units), when compared to hSCs from the CTR group (Figure 5.4a). *PGC1α* is a master regulator of mitochondrial biogenesis in mammals and its mRNA abundance was decreased in hSCs from the groups treated with ACT (0.001 ± 0.0003 arbitrary units) and with SLC-0111 (0.271 ± 0.150 arbitrary units), in comparison with the levels detected in cells from the CTR group (1.021 ± 0.016 arbitrary units) (Figure 5.4b). The *NRF-1* relative mRNA abundance was also significantly reduced when SCs were exposed to the general inhibitor of CA (0.004 ± 0.001 arbitrary units), in relation to the levels detected in cells from the CTR group (1.019 ± 0.016 arbitrary units), but not when only CA IX and CA XII were inhibited by exposure to SLC-0111 (0.30 ± 0.20 arbitrary units) (Figure 5.4c). Hif1-α mRNA abundance was also significantly reduced in hSCs treated with ACT (0.151 ± 0.045 arbitrary units), but not in those treated with the selective CA inhibitor SLC-0111 (0.650 ± 0.240 arbitrary units), in comparison to the levels detected in cells from the CTR group (1.028 ± 0.02 arbitrary units) (Figure 5.4d). mtDNA copy number was also evaluated by quantifying the number of copies of *MT-ND1*, which codifies for a subunit of the protein NADH dehydrogenase 1. This protein is part of mitochondrial complexes, specifically complex I that is responsible for the first step in the electron transport. No alterations were observed in the mtDNA copy number in cells exposed to ACT (840 ± 237.7 arbitrary units) or SLC-0111 (775.4 ± 149.1 arbitrary units) when compared with the levels detected in hSCs from the CTR group (1025 ± 285 arbitrary units) (Figure 5.5a).

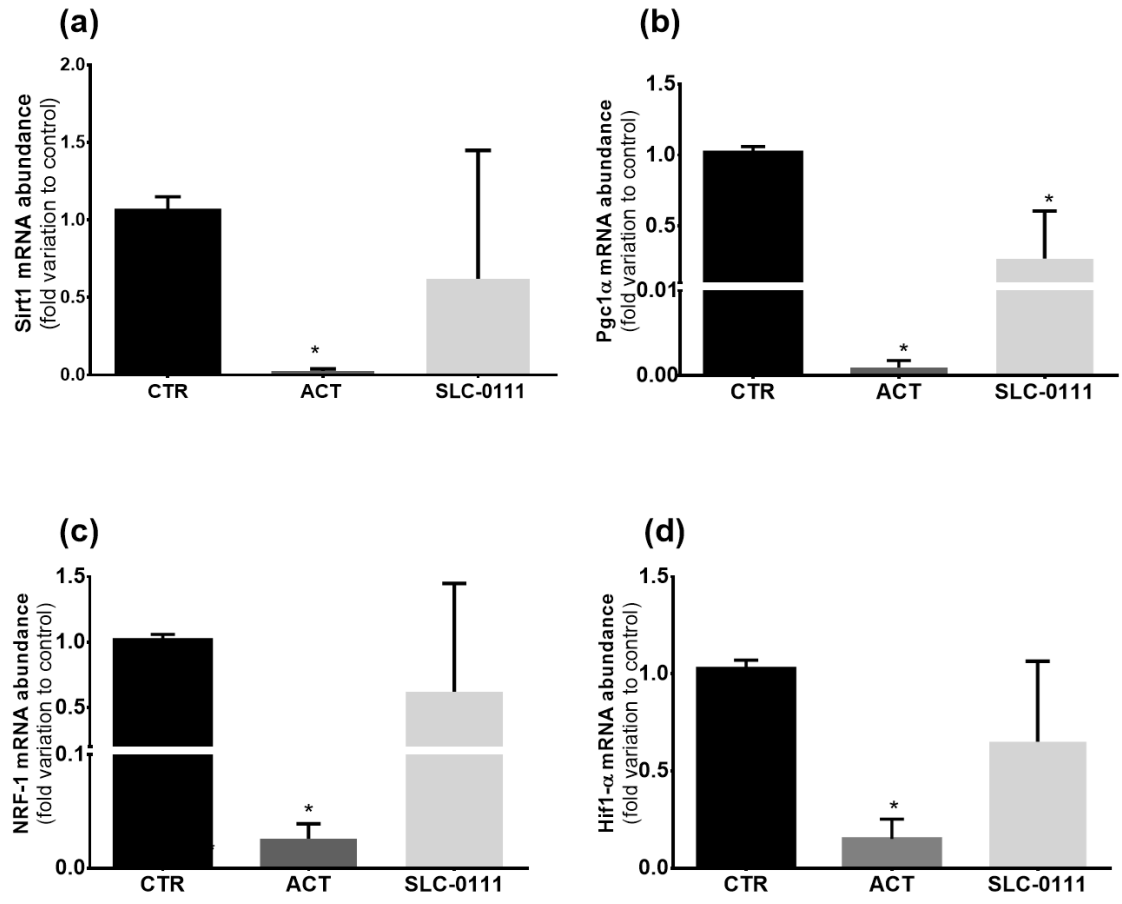


Figure 5.4 Effect of acetazolamide (ACT) and SLC-0111 on: **a)** *Sirtuin 1* (*SIRT1*), **b)** *peroxisome proliferator-activated receptor gamma coactivator 1α* (*PGC1α*), **c)** *Nuclear respiratory factor-1* (*NRF-1*) and **d)** *Hypoxia-inducible factor 1-alpha* (*HIF1-α*) mRNA abundance in human Sertoli cells (hSCs). Results are expressed as mean ± SEM (n = 6 for each condition) and normalized to control group. *Significantly different relative to control ($p<0.05$).

Carbonic anhydrase is not involved on the mitochondrial membrane potential of hSCs

ATP is produced by mitochondria due to a flux electrons resulting from oxidation of substrates that are guided by redox carriers (complexes I, II, III, and IV) and mitochondrial complex V that is linked with proton pumping by an F-ATPase. The analysis of protein levels of OXPHOS complexes showed that exposure of hSCs to ACT does not modulate the levels of any of the complexes. The protein levels of all complexes were not altered when hSCs were treated with ACT in relation to cells in the CTR group as described in Table 5.2.

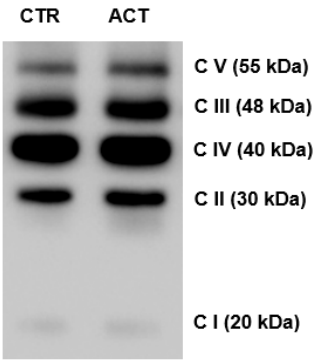
Mitochondrial membrane potential (as determined by the JC-1 ratio) was also not altered when CA was inhibited. JC-1(Aggregate/ Monomers) ratio was 1.14 ± 0.05 (fold variation to control) for cells treated with ACT and 1.00 ± 0.07 for untreated cells (fold variation to control) (Figure 5.5b).

Tabela 5.2 Effect of acetazolamide (ACT) in protein expression levels of mitochondrial complexes of human Sertoli cells (hSCs). **a)** Protein expression levels of mitochondrial complexes in hSCs from the control group and treated with ACT. Results are presented in fold variation to control. **b)** Representative Western Blot experiments.

(a)

Mitochondrial complexes	Control Group	ACT Group
CI	1.00 ± 0.03	1.05 ± 0.19
CII	1.00 ± 0.01	1.46 ± 0.34
CIII	1.00 ± 0.01	1.35 ± 0.11
CIV	1.00 ± 0.02	1.12 ± 0.05
CV	1.00 ± 0.05	1.34 ± 0.08

(b)



Lipid metabolism is promoted by the inhibition of carbonic anhydrase in hSCs

The quantification of lipid accumulation by Oil red O staining is used for any *in vitro* cultured cells that may accumulate triglycerides or cholesteryl esters in the cytoplasm as lipid droplets. We determined the impact of ACT in lipid accumulation in hSCs. The treatment of hSCs during 24 hours with ACT (0.996 ± 0.177-fold variation to control) did not altered lipid accumulation in relation to cells from the CTR group (1.000 ± 0.118 fold variation to control) (Figure 5.5c). Still, the inhibition of CA in hSCs seems to promote lipid degradation by increasing mRNA abundance in HSL when cells were treated with ACT (3.78 ± 1.17-fold variation to control) in comparison to cells from the CTR group (1.00 ± 0.45-fold variation to control) (Figure 5.5d). Other genes related to lipid synthesis and lipid accumulation like *Perilipin 2* (*PLIN2*), *Acyl-CoA synthetase long chain family member 1* (*ACSL1*) and *Acetyl-CoA carboxylase* (*ACC*) were also analysed though gene expression of *PLIN2*, *ACSL1* and *ACC* have not presented alteration after inhibition of CAs in relation to hSCs from the CTR group (data not shown).

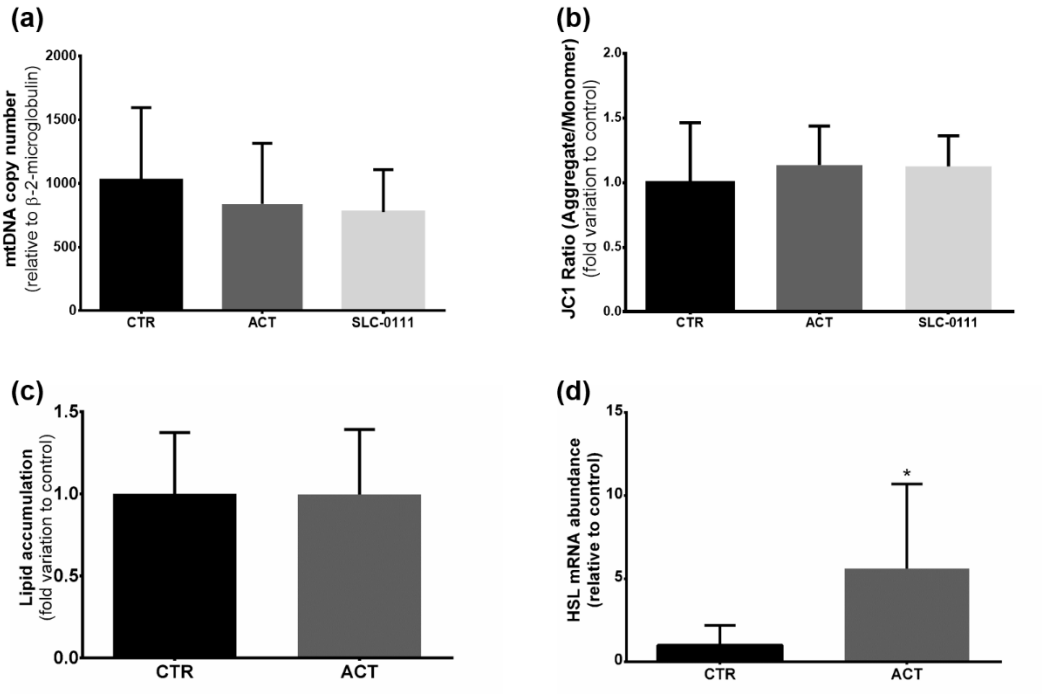


Figure 5.5 Effects of carbonic anhydrase (CA) inhibition. **a)** mtDNA copy number in human Sertoli cells (hSCs) after treatment with acetazolamide (ACT) and SLC-0111 and without any treatment (CTR). Quantification of mtDNA abundance was normalized with β-2-microglobulin. **b)** Effects of ACT and SLC-0111 in mitochondrial membrane potential in hSCs. The figure shows pooled data of independent experiments, indicating JC1 ratio (aggregates/monomers). **c)** Effect of ACT in lipid accumulation in hSCs. Quantification of oil red O staining area was determined using an image analyzer. **d)** Effect of ACT in hormone sensitive lipase (HSL) mRNA abundance in hSCs and in control group (CTR). Results are expressed as mean ± standard error of mean (SEM) (n = 6 for each condition) and normalized to control group. *Significantly different relative to control ($p<0.05$).

Discussion

In the seminiferous tubules, the maintenance of ion homeodynamics of both intracellular and extracellular milieu (specifically in the STF) has been associated with a successful progression of spermatogenesis [15]. Approximately 40% of the seminiferous tubules are filled by SCs [16, 17]. SCs are the “nurse” cells that have an essential role controlling the maintenance of the spermatogenic event by providing the metabolic and ionic needs to the developing germ cells. These cells are responsible for several features of the STF, including its ionic composition and pH [18]. The principal mobile physiological pH buffer is HCO_3^-, which protects the cells from changes in intracellular pH and in luminal fluids [19]. The presence of HCO_3^- transporters in male reproductive tract is clear evidence that this ion is crucial for male reproduction [20-22]. The alterations in HCO_3^- concentration and pH in SCs can be related with disturbances in ionic balance in male reproductive tract and can be a cause for subfertility or infertility [21, 23]. In this work we analysed the effect of reduced HCO_3^- production by inhibition of CAs in key features of the metabolism and physiology of hSCs. We were able to identify for the first time

CA VB, CA IX and XII in hSCs. CA IX and CA XII are two cell membrane-bound isoforms whose expression is frequently altered in many types of tumours. There are studies indicating that these enzymes may contribute to the characteristic microenvironment of the tumour by preserving extracellular acidic pH that is propitious for cancer cells grow and metastasize [24]. The CA V is a mitochondrial isoform that is expressed in two variants A and B, variant CA VA is present only in hepatocytes, whereas CA VB has a much wider tissue distribution [25]. It has been suggested that CA V is important for the regulation of several metabolic pathways like gluconeogenesis, lipogenesis and ureagenesis [26, 27].

In numerous biological processes, CAs influence ion transport and intracellular and extracellular pH [28]. The intracellular pH of hSCs was not altered with the general inhibition of CAs by ACT. These results can be explained by the existence of other physiological buffers that are able to maintain ionic composition in addition to HCO_3^- [29, 30]. Furthermore, we cannot discard a residual contribution of this buffering system, for even when CAs are inhibited it does not totally inhibit the formation of HCO_3^-, instead it highly decreases its production, since CAs are responsible for accelerating the hydration of CO_2 [31]. The expression of HCO_3^- transporters in the membrane of SCs also can participate in achieving the ionic balance between intra and extracellular medium [20].

Contrary to pH, the metabolism of SCs is highly affected by the decreased production of HCO_3^-. Indeed, our data show that inhibition of CAs by ACT causes an increased production of lactate and alanine, without changes in glucose consumption, pyruvate production or intracellular LDH activity in hSCs. LDH is an enzyme that catalyses the interconversion of pyruvate and lactate. One of the major modulators of its action is the stoichiometric pressure that this enzyme is subjected to. For instance, a higher intracellular production of pyruvate leads to a higher production of lactate without an alteration on the global activity of LDH, as observed in our experimental work. Hence, inthese cells, glucose is mostly used by SCs to produce pyruvate that can be then converted in lactate, alanine and acetyl-CoA. Lactate is essential for the development of germ cells. Developing germ cells are unable to metabolize glucose and use the lactate produced by SCs as an energy substrate [32, 33]. In addition, it has been suggested that lactate production can also be obtained by other pathways, although these processes are not well understood.

Our results suggest that glucose-derived pyruvate is mostly converted to lactate and alanine and thus, reduction in the conversion of pyruvate to acetyl-CoA is expected which may end-up in compromised mitochondria dynamics. However, the inhibition of CAs did not cause changes in the expression of the electron transport chain complexes or in mitochondria DNA copies. Still, inhibition of CAs by ACT could alter mitochondrial function, for instance, by promoting a consequent decrease in the formation of HCO_3^-, which is detrimental for the carboxylation of

pyruvate to oxaloacetate, an important anaplerotic reaction under the catalytic influence of mitochondrial isoenzymes (CA V) [6]. Hence, we investigated the impact of ACT on some aspects of mitochondrial dynamics. Several factors directly regulate mitochondrial genes and control mitochondrial biogenesis [34]. We observed a decrease in the expression of genes involved with mitochondrial biogenesis when all CAs were inhibited by ACT in hSCs. The primary regulator of the coordinated transcription of the mitochondrial and nuclear genome during mitochondrial biogenesis is *PGC1α* that is reduced by more than 99% when CAs are inhibited in hSCs. As we identified three CA isoforms in hSCs, to scrutinize if this action is mediated by the membrane isoforms or mitochondrial isoform, we used a specific inhibitor of CA IX and CA XII (SLC-0111). When cells were exposed to SLC-0111 we also observed a decreased abundance of *PGC1α* mRNA. This indicates that both isoforms may play a role in mitochondrial biogenesis. Moreover, it is known that through a functional directly interaction, SIRT1 regulates the activity of the one most important metabolic transcriptional co-activators of genes involved in regulation of metabolism and mitochondrial biogenesis, the PGC1α [35, 36]. PGC1α is also an upstream inducer of genes of mitochondrial metabolism by regulation the activity of some hormone nuclear receptors and nuclear transcription factors (NRF-1 and 2). NRF-1 controls many mitochondrial genes, is a downstream effector of SIRT1/ PGC1α and activates the nuclear genes coding for subunits of the oxidative phosphorylation (OXPHOS), expression of mitochondrial transporters and ribosomal proteins [37, 38]. Moreover, SIRT1 also has an important role in deacetylating and repressing the activity of HIF-1α, which is known to down-regulate mitochondrial function and oxygen consumption [39]. The mRNA abundance of all these players, *SIRT1*, *NRF-1* and *HIF-1α*, exhibited a decrease when hSCs were exposed to the inhibitor of all CAs, but no differences were observed when only CA IX and CA XII were inhibited using SLC-0111. These results illustrate that the mitochondrial isoform has an important role in controlling the expression of key genes related to mitochondrial biogenesis. Contrastingly, no effect was observed on mitochondria membrane potential of hSCs after inhibition of all CAs. Thus, ACT does not affect the mitochondrial membrane potential of hSCs, but rather genes responsible for its biogenesis.

Inhibitors of CAs are already used for the treatment of some metabolic diseases, as is the case of obesity, due to their interaction with lipogenesis. Scozzafava and collaborators [27] described in their review that CAs, namely the mitochondrial isoforms, are implicated in the *novo* lipogenesis. Therefore, they are used as targets in cases of lipid metabolism deregulation, namely antiobesity effects. In hSCs, the inhibition of CAs also caused alterations in lipid metabolism, as showed by the increased abundance of *HSL* in these cells. Durham and Grogan [40] described the presence of cholesterol ester hydrolases in testis. These cholesterol ester hydrolases exhibit the typical biochemical properties of HSL, including the typical feature

of HSL of being phosphorylated and activated by cAMP-dependent protein kinase [41]. The role of HSL in testis and SCs is not yet well understood. In other tissues, like adipose tissue, HSL is involved in steroidogenesis by providing free cholesterol for steroid synthesis [42, 43]. Testes store triacylglycerols and cholesterol together with phospholipids in intracellular lipid droplets. These stores are important for steroid biosynthesis and spermatogenesis. However, HSL is not present in Leydig cells (the cells responsible for steroid synthesis), but rather in SCs and in germ cells [44]. It is known that HSL knockout mice males are sterile, presenting oligoospermia, and their testes contain increased amounts of cholesterol esters [45]. Thus, it is possible that HSL is not involved in steroidogenesis and plays another role in reproductive function, as it was speculated that cholesterol or fatty acids released by the action of HSL are required for spermatogenesis. We hypothesize that HSL may be involved in the production of substrates for β-oxidation of fatty acids in SCs and these molecules undergo oxidative phosphorylation via the tricarboxylic acid (TCA) to produce malate. Like in adipose tissue, malate can be converted in pyruvate by the action of malic enzymes [46, 47]. Although little is known about the significance of this specific pathway on SCs physiology, it has been reported that it can serve as an intracellular source for the production lactate under specific conditions [46, 47]. In fact, it has been described that CAs may play an important role in lipid utilization under certain conditions [27, 48, 49].

In summary, our results suggest that CAs and consequently HCO_3^- are essential for hSCs physiological functions that sustain spermatogenesis. The inhibition of CAs, particularly isoform VB proved to affect cell metabolism, and the expression of genes involved in mitochondrial biogenesis and lipids metabolism in hSCs, which may compromise spermatogenesis.

References

1. Boron, W.F., 2004, Regulation of intracellular pH. Advances in physiology education, 28(4): p. 160-179.
2. Supuran, C.T., 2008, Carbonic anhydrases-an overview. Current Pharmaceutical Design, 14(7): p. 603-614.
3. Swietach, P., et al., 2010, New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. Oncogene, 29(50): p. 6509.
4. Supuran, C.T. and A. Scozzafava, 2000, Carbonic anhydrase inhibitors and their therapeutic potential. Expert Opinion on Therapeutic Patents, 10(5): p. 575-600.

5. Nishimori, I., et al., 2007, Carbonic anhydrase inhibitors. DNA cloning, characterization, and inhibition studies of the human secretory isoform VI, a new target for sulfonamide and sulfamate inhibitors. Journal of Medicinal Chemistry, 50(2): p. 381-388.
6. Supuran, C.T., 2008, Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nature Reviews Drug Discovery, 7(2): p. 168.
7. Supuran, C.T., A. Scozzafava, and A. Casini, 2003, Carbonic anhydrase inhibitors. Medicinal Research Reviews, 23(2): p. 146-189.
8. Griswold, M.D., 1998, The central role of Sertoli cells in spermatogenesis. in Seminars in cell & developmental biology, 9(4): p. 411-416.
9. Fisher, D., 2002, New light shed on fluid formation in the seminiferous tubules of the rat. The Journal of physiology, 542(2): p. 445-452.
10. Richburg, J.H., D.M. Redenbach, and K. Boekelheide, 1994, Seminiferous tubule fluid secretion is a Sertoli cell microtubule-dependent process inhibited by 2, 5-hexanedione exposure. Toxicology and Applied Pharmacology, 128(2): p. 302-309.
11. Levine, N. and D.J. Marsh, 1971, Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. The Journal of physiology, 213(3): p. 557-570.
12. Klatte, T., et al., 2007, Carbonic anhydrase IX (CAIX) in bladder cancer: a diagnostic, prognostic and therapeutic molecular marker. Journal of Urology, 115(7): p.1448-1458.
13. Türeci, Ö., et al., 1998, Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. Proceedings of the National Academy of Sciences, 95(13): p. 7608-7613.
14. Shah, G.N., et al., 2000, Mitochondrial carbonic anhydrase CA VB: differences in tissue distribution and pattern of evolution from those of CA VA suggest distinct physiological roles. Proceedings of the National Academy of Sciences, 97(4): p. 1677-1682.
15. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. Journal of Membrane Biology, 236(2): p. 215-224.
16. de França, L.R., et al., 1993, Surface and surface-to-volume relationships of the Sertoli cell during the cycle of the seminiferous epithelium in the rat. Biology of Reproduction, 49(6): p. 1215-1228.
17. Sharpe, R.M., et al., 2003, Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction, 125(6): p. 769-784.
18. Pastor-Soler, N., C. Piétrement, and S. Breton, 2005, Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. Physiology, 20(6): p. 417-428.

19. Bernardino, R.L., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current Medicinal Chemistry, 20(32): p. 4037-4049.
20. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.
21. Bernardino, R.L., et al., 2013, Effect of prediabetes on membrane bicarbonate transporters in testis and epididymis. Journal of Membrane Biology, 246(12): p. 877-883.
22. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and Cellular Biochemistry, 408(1-2): p. 47-54.
23. Breton, S., et al., 1996, Acidification of the male reproductive tract by a proton pumping (H+)-ATPase. Nature Medicine, 2(4): p. 470.
24. Ivanov, S., et al., 2001, Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. American Journal of Pathology, 158(3): p. 905-919.
25. Nishimori, I., et al., 2005, Carbonic anhydrase inhibitors. The mitochondrial isozyme VB as a new target for sulfonamide and sulfamate inhibitors. Journal of Medicinal Chemistry, 48(24): p. 7860-7866.
26. Sly, W.S. and P.Y. Hu, 1995, Human carbonic anhydrases and carbonic anhydrase deficiencies. Annual Review of Biochemistry, 64(1): p. 375-401.
27. Scozzafava, A., C.T. Supuran, and F. Carta, 2013, Antiobesity carbonic anhydrase inhibitors: a literature and patent review. Expert Opinion on Therapeutic Patents, 23(6): p. 725-735.
28. Wykoff, C.C., et al., 2001, Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast. American Journal of Pathology, 158(3): p. 1011-1019.
29. Roos, A. and W.F. Boron, 1981, Intracellular pH. Physiological Reviews, 61(2): p. 296-434.
30. Oliveira, P., et al., 2009, Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. Journal of Membrane Biology, 227(1): p. 49-55.
31. Geers, C. and G. Gros, 2000, Carbon dioxide transport and carbonic anhydrase in blood and muscle. Physiological Reviews, 80(2): p. 681-715.
32. Oliveira, P.F., et al., 2015, The Warburg effect revisited—lesson from the Sertoli cell. Medicinal Research Reviews, 35(1): p. 126-151.
33. Dias, T.R., et al., 2013, Glucose transport and metabolism in sertoli cell: relevance for male fertility. Current Chemical Biology, 7(3): p. 282-293.
34. Wu, Z., et al., 1999, Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell, 98(1): p. 115-124.

35. Nemoto, S., M.M. Fergusson, and T. Finkel, 2005, SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1α. Journal of Biological Chemistry, 280(16): p. 16456-16460.
36. Knutti, D. and A. Kralli, 2001, PGC-1, a versatile coactivator. Trends in Endocrinology & Metabolism, 12(8): p. 360-365.
37. Finkel, T., 2006, Cell biology: a clean energy programme. Nature, 444(7116): p. 151.
38. Aquilano, K., et al., 2010, Peroxisome Proliferator-activated receptor γ Co-activator 1α (PGC-1α) and Sirtuin 1 (SIRT1) reside in mitochondria possible direct function in mitochondrial biogenesis. Journal of Biological Chemistry, 285(28): p. 21590-21599.
39. Papandreou, I., et al., 2006, HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metabolism, 3(3): p. 187-197.
40. Durham, L.A. and W. Grogan, 1984, Characterization of multiple forms of cholesteryl ester hydrolase in the rat testis. Journal of Biological Chemistry, 259(12): p. 7433-7438.
41. Hoffmann, A.-M., A. Bergh, and T. Olivecrona, 1989, Changes of testicular cholesteryl ester hydrolase activity in experimentally cryptorchid rats. Journal of Reproduction and Fertility, 86(1): p. 11-18.
42. Cook, K.G., et al., 1983, Cytosolic cholesterol ester hydrolase from bovine corpus luteum: Its purification, identification, and relationship to hormone-sensitive lipase. Biochimica et biophysica acta, 752(1): p. 46-53.
43. Cook, K.G., et al., 1982, Direct Evidence that Cholesterol Ester Hydrolase from Adrenal Cortex is the Same Enzyme as Hormone-Sensitive Lipase from Adipose Tissue. European Journal of Biochemistry, 125(1): p. 245-249.
44. Holst, L.S., et al., 1994, Localization of hormone-sensitive lipase to rat Sertoli cells and its expression in developing and degenerating testes. FEBS Letters, 355(2): p. 125-130.
45. Osuga, J.-i., et al., 2000, Targeted disruption of hormone-sensitive lipase results in male sterility and adipocyte hypertrophy, but not in obesity. Proceedings of the National Academy of Sciences, 97(2): p. 787-792.
46. Sauer, L., et al., 1980, Mitochondrial malic enzymes. Mitochondrial NAD (P)+-dependent malic enzyme activity and malate-dependent pyruvate formation are progression-linked in Morris hepatomas. Journal of Biological Chemistry, 255(9): p. 3844-3848.
47. Wise, E.M. and E.G. Ball, 1964, Malic enzyme and lipogenesis. Proceedings of the National Academy of Sciences, 52(5): p. 1255-1263.
48. Côté, C.H., F. Ambrosio, and G. Perreault, 1999, Metabolic and contractile influence of carbonic anhydrase III in skeletal muscle is age dependent. American Journal of Physiology, 276(2): p. R559-R565.

49. Dorai, T., et al., 2006, Role of carbonic anhydrases in the progression of renal cell carcinoma subtypes: proposal of a unified hypothesis. Cancer Investigation, 24(8): p. 754-779.
50. Bernardino, R.L., M.G. Alves, and P.F. Oliveira, Establishment of Primary Culture of Sertoli Cells, in Sertoli Cells: Methods and Protocols, M.G. Alves and P.F. Oliveira, Editors. 2018, Springer New York: New York, NY. p. 1-8.
51. Pan, J., et al., 2014, Synthesis and evaluation of 18F-labeled carbonic anhydrase IX inhibitors for imaging with positron emission tomography. Journal of Enzyme Inhibition and Medicinal Chemistry, 29(2): p. 249-255.
52. Pacchiano, F., et al., 2011, Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. Journal of Medicinal Chemistry, 54(6): p. 1896-1902.
53. Alves, M.G., et al., 2012, In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1823(8): p. 1389-1394.
54. Oliveira, P.F., et al., 2009, Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction, 137(2): p. 353-359.
55. Alves, M.G., P.J. Oliveira, and R.A. Carvalho, 2011, Substrate selection in hearts subjected to ischemia/reperfusion: role of cardioplegic solutions and gender. NMR in Biomedicine, 24(9): p. 1029-1037.
56. Pfaffl, M.W., 2001, A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research, 29(9): p. e45-e45.

Chapter 6

Estradiol modulates Na^+-dependent HCO_3^- transporters altering intracellular pH and ion transport in human Sertoli cells: a role on male fertility?

This chapter was adapted from the published work:

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Estradiol modulates Na^+-dependent HCO_3^- transporters altering intracellular pH and ion transport in human Sertoli cells: a role on male fertility?

Abstract

Infertile men often present deregulation of serum estrogen levels. Notably, high levels of estradiol (E2) are associated with low sperm production and quality. Sertoli cells (SCs) are responsible for spermatogenesis maintenance and are major targets for the hormonal signalling that regulates this complex process. In this study, we used primary cultures of human SCs and studied the localization, expression and functionality of the Na^+-dependent HCO_3^- transporters by confocal microscopy, immunoblot, epifluorescence and voltage-clamp, after 24 hours of exposure to E2 (100 nM). All studied transporters were identified in human SCs. In E2-treated human SCs there was an increase in NBCn1, NBCe1 and NDCBE protein levels, as well as an increase in intracellular pH and a decrease on transcellular transport. We report an association between increased levels of E2 and the expression/function of Na^+-dependent HCO_3^- transporters in human SCs. Our results provide new evidence on the mechanisms by which E2 can regulate SCs physiology and consequently spermatogenesis. These mechanisms may have an influence on male reproductive potential and help to explain male infertility conditions associated with estrogen deregulation. Exposure to E2 increased human SCs intracellular pH. E2 is a modulator of ionic transcellular transport in human SCs.

Keywords: Electron/light microscopy, Membrane transport, Reproductive biology, Transporters.

Introduction

Infertility affects millions of couples and almost one third of the cases results from a male fertility defect [1]. Up to 75% of all male infertility cases are of unknown causal factor (idiopathic infertility), with patients presenting unexplained abnormalities in reproductive parameters [2]. Deregulation of serum estrogen levels have been observed in numerous infertile men and high levels of estradiol (E2) have been associated with low sperm production and quality [3, 4]. Several studies provided evidence that E2 plays a key role in spermatogenesis and male reproductive function (for review see [5]). Despite the mechanisms modulated by E2 remain a matter of debate, data suggests that this hormone exerts a variety of effects on testicular cells, via their specific receptors [6-9]. In fact, estrogen receptor knockout animal models present compromised spermatogenesis, steroidogenesis and male fertility [10, 11]. E2 has also been

implicated in the regulation of ion transporters expression in the male reproductive ducts, influencing Na^+ reabsorption and passive water transport [12]. These findings have shown that estrogens can modulate membrane ion transporters expression and functionality in the male reproductive tract, which is crucial to the control of the luminal fluid composition. Still, the impact of hyperestrogenism is not yet fully elucidated, being imperative to identify the key mechanisms altered due to high levels of E2 and its consequences on male fertility.

Cell physiology is dependent on the functioning of multiple cellular processes that only occur over a narrow pH range. In fact, the maintenance of the extracellular (pHo) and intracellular (pHi) pH is a process of paramount importance. The HCO_3^- system is primarily known as the central buffer of the extracellular fluids, but it has also been consistently reported that it plays a major role on pHi maintenance in numerous types of cells [13-15]. Cells possess in their plasmatic membrane a range of ion transporters that participate in pH regulation. In humans, the solute carrier 4 (SLC4) transporters are known as the main HCO_3^--transporter family (for review, see [16]), facilitating the exchange of HCO_3^- across the plasma membrane of mammalian cells together with Cl^-, Na^+ or $B(OH)_4^-$. These mechanisms decisively contribute to the regulation of cell volume and secondarily to membrane potential [17]. Half of these transporters rely on Na^+ and, due to the physiological relevance of this particular ion electrochemical gradient, Na^+-coupled HCO_3^- transporters (NCBTs) play a specialized role in the maintenance of both, pHi and whole-body pH, as well as to the transepithelial transport of HCO_3^- [18].

The control of pHi and pHo along the reproductive tract is essential for male reproductive health. The concentration of HCO_3^- plays a key role in the reproductive physiology and the SLC4 family members are pivotal due to their involvement in HCO_3^- homeodynamics (for review, see [16]). The composition and regulation of the seminiferous tubular fluid (STF) pH are essential for male fertility preservation [19, 20]. The Sertoli cells (SCs) are located within the seminiferous epithelium and form the Sertoli cell barrier, most commonly known as blood-testis barrier (BTB). The microenvironment created by these cells influences the development of germ cells and all accountable processes for the formation of viable sperm [21, 22]. In male reproductive tract, SCs are major targets for the hormonal signalling that regulates spermatogenesis [16, 23, 24] particularly by sex steroid hormones (androgens and estrogens) (for review, see [16]). Herein, we aimed to identify the cellular localization of NCBTs. We selected three key NCBTs (NBCe1, NBCn1 and NDCBE) of the SLC4 family, already identified in testicular tissue [25] and evaluated the effect of high levels of E2 on the expression and function of these transporters in human SCs.

Material and Methods

Chemicals

Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany), Tween 20 from Applichem (Darmstadt, Germany) and dried milk from Regilait (Saint-Martin-Belle-Roche, France). Pluronic F-127, nigericin, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), Alexa Fluor 680 goat anti-rabbit IgG, Alexa Fluor 680 rabbit anti-goat IgG and Hoechst 33342 dye were purchased from Invitrogen (Molecular Probes, Carlsbad, USA). All other antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Dulbecco's modified eagle medium, Ham's nutrient mixture F12 (DMEM: Ham's F12), E2, bovine serum albumin (BSA), Trypsin-EDTA, Insulin-Transferrin-Selenium supplement (ITS supplement), ATP, DIDS and all other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise.

Patient's selection, ethical issues and testicle tissue preparation

Testicular biopsies were obtained from patients seeking for fertility treatment for recovery of male gametes and used after informed written consent. In the present study only cells left in the tissue culture plates after patients' treatment were used. Human SCs were isolated from six testicular biopsies selected from patients with conserved spermatogenesis and seeking for treatment due to anejaculation (psychological, vascular, neurologic), vasectomy or traumatic section of the vas deferens. Each testicle biopsy was collected in sperm preparation medium (SPM-Hepes buffer; Medicult, Florence, Italy) and kept at 33°C with 6% CO_2 in air until use.

Primary culture of human Sertoli cells

All studies were performed according to the Declaration of Helsinki. The clinical study of the patients and testicular tissue processing was performed at the Centre for Reproductive Genetics Prof. Alberto Barros (Porto, Portugal) in accordance with the Guidelines of the Local, National and European Ethical Committees. Testicular biopsies were washed twice in ice-cold Hank's balanced salts solution (HBSS) (in g/L: potassium chloride 0.4, potassium phosphate monobasic anhydrous 0.06, sodium chloride 8, sodium phosphate dibasic 0.045, D-glucose 1 , sodium bicarbonate 0.35 and pH 7.4) by centrifuging at 500xg, room temperature during 5 minutes. Human SCs were isolated by a method previously described by Oliveira and collaborators [20]. The resulting cellular pellet was suspended in Sertoli culture medium, which consisted of a 1:1 mixture of DMEM: Ham F12, supplemented with 15 mM HEPES, 50 U/mL penicillin, 50 mg/mL streptomycin sulphate, 0.5 mg/mL fungizone, 50 µg/mL gentamicin and 10% inactivated FBS. In order to disaggregate large SC clusters, the cellular suspension was

forced though a 20G needle. For cell culture, the concentration was adjusted to 5000 clusters/mL in Sertoli culture medium, plated on 75 cm^2 culture flasks (Cell+; Sarstedt, Nümbrecht, Germany), and incubated at 33ºC in an atmosphere of 6% CO_2: 94% O_2.

Experimental groups

Human SCs were grown until reaching a confluence of 90%–95%. Then the culture medium was replaced by serum and phenol-red free medium (DMEM: F12 supplemented with Insulin-transferrin-selenium supplement (ITS; in mg/L: insulin 10, transferrin 5.5, selenium 0.0067), pH 7.4. The cells were divided in two groups: a control group without E2 and a group treated with E2 (100 nM). The E2 concentration was chosen based on published data, which reported that intratesticular interstitial fluid concentrations of this hormone are notably higher than those of circulating plasma, reaching values up to 200 nM [30, 37]. Control groups were treated with same amount of solvent (ethanol) used in E2 group (<0.025% v/v). Our group and others tested the ethanol dose used, and the results showed that it does not alter the conditions of cultured SCs [38, 39]. Treatments were performed during 24 hours in an atmosphere of 6% CO_2, 94% O_2 at 33ºC. At the end of the treatment, the total number of cells per flask was determined with a Neubauer chamber and cells were collected using a Trypsin-EDTA solution for protein extraction.

Immunofluorescence

SCs obtained from primary cultures were seeded into Snapwell cell culture inserts with a polyester membrane of 0.4 µm pore size (Corning, Tewksbury, USA) and maintained in Sertoli culture medium at 33ºC in an atmosphere of 6% CO_2: 94% O_2, until reaching confluence. Confluence was assessed by measuring transepithelial resistance and experiments were undertaken only when the cell monolayer had developed a transepithelial resistance ranging from 100 to 300 $\Omega.cm^2$, as described [40]. Confluent monolayers of SCs were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were blocked with 20% FBS in PBS and 0.1% Tween-20 for 1 hour at room temperature. Cells were incubated with the primary antibodies, goat anti-SLC4A4 (1:100, Sc-162214), goat anti- SLC4A8 (1:100, Sc-169346) and rabbit anti-SLC4A7 (1:200, Sc-99633), overnight at 4ºC. Afterwards, cells were washed and incubated 1 hour, at room temperature, with Alexa Fluor 680 goat anti-rabbit IgG (1:1000, A-20188) or Alexa Fluor 680 rabbit anti-goat IgG (1:1000, A-21086). The nuclei of cells were stained with Hoechst 33342 dye (1:1000, H-1399), 10 minutes, at room temperature. Fluorescence was observed by confocal microscopy in a Zeiss LSM 510 Meta system (Zeiss Imaging Systems, Gottingen, Germany), to evaluate the adluminal or basal localization of SLC4A4, SLC4A8 and SLC4A7 in human cultured SCs.

Immunoblotting

Protein expression was evaluated by the Slot-Blot technique, as previously described [41, 42]. In brief, total proteins were extracted from human SCs using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA). Protein concentration was determined by the Bio-Rad Bradford micro-assay (Bio-Rad, Richmond, USA). Protein samples (5.0 µg) were diluted in PBS to a final volume of 100 µL. Activated polyvinylidene difluoride (PVDF) membranes were used. The slot-blot technique was performed using a Hybri-slot manifold system (Biometra, Göttingen, Germany). Membranes were blocked by incubation during 90 minutes with 5% non-fat milk. The resulting membranes were incubated with goat anti-SLC4A4 (1:500, Sc-162214), goat anti-SLC4A8 (1:1000, Sc-169346) and rabbit anti-SLC4A7 (1:500, Sc-99633). The immunoreactive proteins were detected separately with donkey anti-goat IgG-AP (1:5000, Sc-2020) or goat anti-rabbit IgG-AP (1:5000, Sc-2007). Prior to the immunoblotting of PVDF membranes, the specificity of the antibodies used was evaluated by western blot analysis as previously described [25]. Only antibodies that specifically stained for the targeted proteins, as described by Bernardino and collaborators [25], were used. Membranes were reacted with ECF detection system and the densities from each band were obtained using the Quantity One Software (Bio-Rad, Hertfordshire, UK). The results are presented as the relative variation in comparison with the control group.

Intracellular pH

SCs were seeded in black polystyrene 96-well plate, until reaching a confluence of 90%–95%. The cells were then treated with E2 or 0,025% ethanol (control group) during 24 hours, as described above. pHi was measured with a fluorescent probe using the method described by Oliveira and collaborators [20]. In brief, SCs were loaded with a fluorescent probe during 15 minutes at 37ºC with Sertoli Control Solution (in mM: NaCl 118; KCl 4.7; CaCl2 2.5; MgCl2 1.8; KH2PO4 1.8; NaHCO3 25; Glucose 14; HEPES 25; pH 7.4) containing 10 µM of BCECF–AM and 0.2% of Pluronic F127. The fluorescence intensities, excited at 490 and 440 nm (fixed emission at 535 nm), were continuously measured with a fluorescence reader plate (SpetraMax Gemini EM, Molecular Devices, Sunnyvale, USA), while the cells were perfused with different solutions. Background fluorescence was determined at the end of each experiment. The background fluorescence intensity was measured for each wavelength. The fluorescence ratios (F490/F440) were calculated after subtracting the background fluorescence intensities for each measurement at each wavelength. Probe calibration was performed at the end of each protocol calibration procedure by the method described by Thomas [43], using solutions of known pH (5.5, 7.4, 8 and 9.0) to which nigericin (10 mM) was

added. To convert the measured fluorescence ratio (F490/F440) to pHi values, the equation described by Oliveira and collaborators [19] was used. The pK of BCECF had an average value of 7.01 ± 0.21 in our experimental conditions.

In order to evaluate the impact of E2 on intracellular H^+ dynamics, changes in pHi were determined (during 15 minutes at 37ºC) in SCs from the different experimental groups, following the addition of ATP (2 mM).

Voltage-Clamp

Human SCs obtained from primary cultures were seeded into Snapwell cell culture inserts with a polyester membrane of 0.4 µm pore size (Corning, Tewksbury, USA) and maintained in Sertoli culture medium at 33ºC in an atmosphere of 6% CO_2: 94% O_2, until reaching confluence, as described above. The cells were then treated with E2 or 0,025% ethanol (control group) during 24 hours, as described above. Afterwards, 1.13 cm2 Snapwell inserts were mounted in Ussing type chambers (Vertical diffusion chamber system, Navicyte, Harvard Apparatus, Massachusetts, USA). Sertoli Control solution (in mM: NaCl 118; KCl; $CaCl_2$ 2.5; $MgCl_2$ 1.8; KH_2PO_4 1.8; $NaHCO_3$ 25; Glucose 14; HEPES 25; pH=7.4) was added to each side of the preparation and the current was left to stabilize. The preparation was kept short-circuited throughout the experiments by means of an electronic voltage-clamp system (Model VCC MCx, Physiologic Instruments, San Diego, CA) and the values of the instantaneous currents were acquired at a sampling rate of 1.min-1. Data was acquired using a personal computer and the software Acquire & Analyze (Physiologic Instruments, Version 2.3). After a steady state period of at least 10 minutes, DIDS (0.5 mM) was added or not to the basal side of the preparation. After 10 minutes of this addition, ATP (2 mM) was added to the adluminal side of the preparation and the short-circuit current (Isc) was followed for a period up to 2 hours. Isc was plotted as the average (± SEM) of the fractional values calculated by dividing each value by the initial Isc value (acquired at time zero), as previously described [44]. Transepithelial potential (Vt) values were calculated as the average of the 10 initial readings or the average of the last 10 readings of each experiment. In this preparation the current/voltage (I/V) curve is linear at least between -100 and +100 mV.

Statistical analysis

The statistical significance of the variation among the experimental groups was assessed by a T-test or a one-way ANOVA. All experimental data are shown as mean ± SEM (n=6 for each condition). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, USA). $P<0.05$ was considered significant.

Results

Three Na^+-dependent HCO_3^- transporters of the SLC4 family are expressed in human Sertoli cells

In this experimental work we evaluated the expression of three NCBTs, members of the SLC4 family: NBCe1 (or SLC4A4), NBCn1 (or SLC4A7) and NDCBE (or SLC4A8). The presence of these transporters was investigated by immunofluorescence. After the cells reached confluence, NBCe1, NBCn1 or NDCBE were detected with specific primary antibodies and adequate fluorescent secondary antibodies (red) and the nuclei were stained with Hoechst 33342 dye (blue). We were able to positively identify, for the first time in isolated cultured human SCs, the presence of the three NCBTs of the SLC4 family. We identified the presence of NDCBE (Figure 6.1a), NBCn1 (Figure 6.1b) and NBCe1 (Figure 6.1c) in human cultured SCs by confocal microscopy. No staining was observed in negative controls.

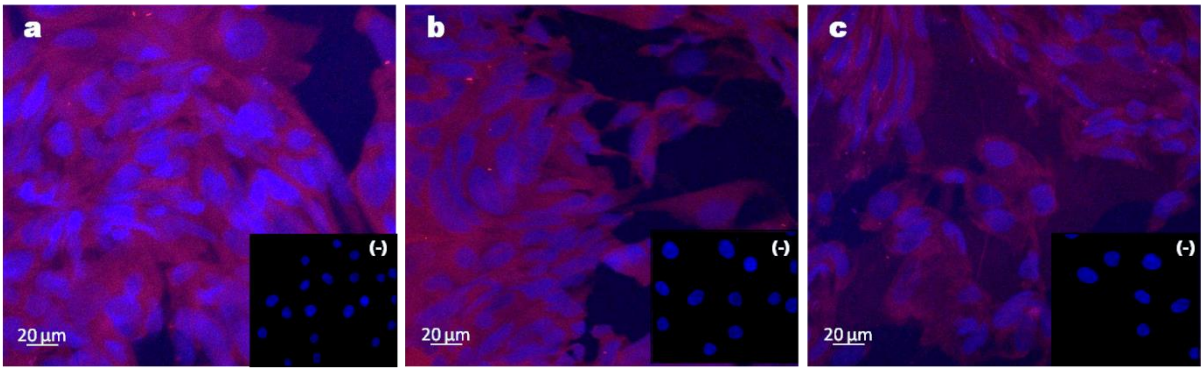

Figure 6.1 Identification of NDCBE, NBCn1 and NBCe1 in human Sertoli cells (SCs). (**a-c**) show the identification of NDCBE (**a**), NBCn1(**b**) and NBCe1(**c**) in human SCs by means of the immunocytochemistry using specific antibodies. (-) represent negative control, without secondary antibody. The nuclei of cells were stained blue with Hoechst 33342 dye. These panels have resulted from cross-sections of the images obtained by confocal microscopy.

NDCBE is localized in the adluminal portion, while NBCn1 and NBCe1 are localized in the basal portion of human Sertoli cells plasma membrane

After the identification of NDCBE, NBCn1 and NBCe1 in cultured human SCs, we further investigated the subcellular localization of these HCO_3^- transporters, to determine if they are localized in the adluminal or basal portions of polarized human SCs membrane. For that purpose, human SCs were cultured in semi-permeable cell culture inserts until reaching confluence. SCs grown under these conditions are reported to develop functional and morphological characteristics of polarized epithelial cells [26]. Subsequently, we evaluated the cellular distribution of the three NCBTs by confocal microscopy. Confocal fluorescence images were acquired using specific primary antibodies and adequate fluorescent secondary antibodies.

Exposure of intact polarized monolayers to an antibody against the human NDCBE demonstrated the presence of this HCO_3^- transporter in the adluminal region of the membrane of human SCs, while no staining was observed in the basal portion of the membrane (Figure 6.2a). Contrastingly, when the polarized human SCs were incubated with the antibodies against the electrogenic NBCe1 and the electroneutral NBCn1 transporters, staining was only observed in the basal membrane, indicating that these transporters are located on the basal portion of the plasma membrane of these cells (Figure 6.2b-c).

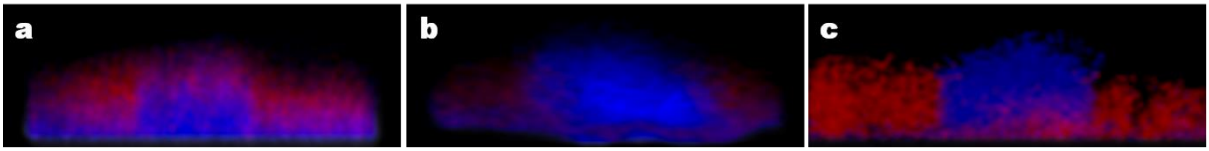

Figure 6.2 Localization of NDCBE, NBCn1 and NBCe1 in human Sertoli cells (SCs). (**a-c**) show the adluminal localization of NDCBE (**a**), and basal localization of NBCn1 (**b**) and NBCe1 (**c**) in polarized epithelial monolayers of human SCs. The nuclei of cells were stained blue with Hoechst 33342 dye. These panels have resulted from cross-sections of the images obtained by confocal microscopy.

Exposure to E2 increased the expression of NDCBE, NBCn1 and NBCe1 in human Sertoli cells

The protein expression levels of the three NCBTs were evaluated in cultured human SCs after incubation with E2, using the immunoblot technique. The protein levels of the three NCBTs exhibited an increase when SCs were treated with E2, as compared to the cells from the control group (Figure 6.3). In human SCs treated with E2 we observed an increase in the protein levels of NDCBE (1.34 ± 0.05 fold variation to control, $P<0.0001$), NBCn1 (1.48 ± 0.12 fold variation to control, $P=0.0025$) and NBCe1 (2.17 ± 0.09 fold variation to control, $P<0.0001$) transporters as compared with cells from the control group (Figure 6.3).

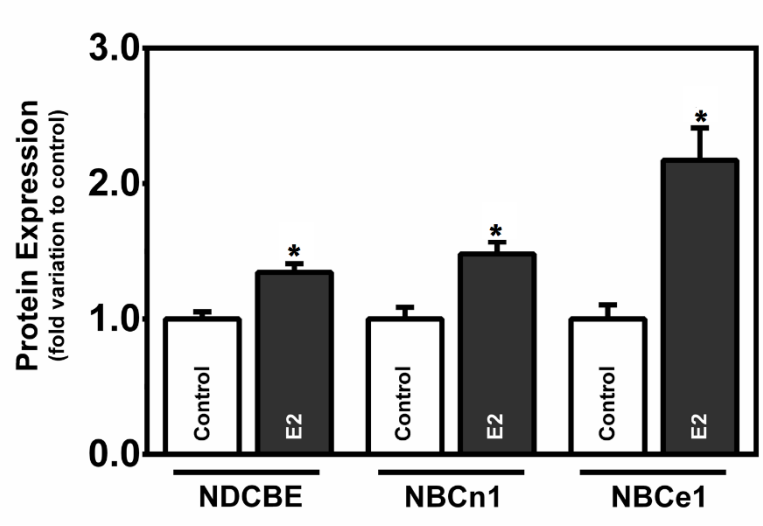

Figure 6.3 Effect of 17β-estradiol (E2) on NDCBE, NBCn1 and NBCe1 protein expression in human Sertoli cells (SCs). Results are expressed as means ± SEM (n=6 for each condition). Significantly different results (T-test, p< 0.05) are indicated: * = vs to control group.

Exposure to E2 increased human Sertoli cells Intracellular pH

Using the H^+-sensitive fluorescent probe BCECF, we determined that human SCs under control conditions (extracellular pH=7.4) exhibited a mean pHi value of 7.06 ± 0.03. When these cells were exposed to E2 we observed that the pHi was increased (7.15 ± 0.02), as compared with SCs from the control group (Figure 6.4a, P=0.0163).

SCs possess functional purinoreceptors that have been implicated in the regulation of ion homeodynamics. Thus, we further evaluated the influence of ATP on intracellular H+ concentration. In fact, exposure of human SCs to ATP caused an increase on this parameter. In SCs from the control group we observed an increase of 0.05 ± 0.01 units in pHi after ATP stimulation (Figure 6.4b). Notably, exposure to E2 before stimulation with ATP, caused an increased shift on pHi of 0.09 ± 0.01 units when compared with the data observed in SCs cultured in control conditions (Figure 6.4b, P<0.0001).

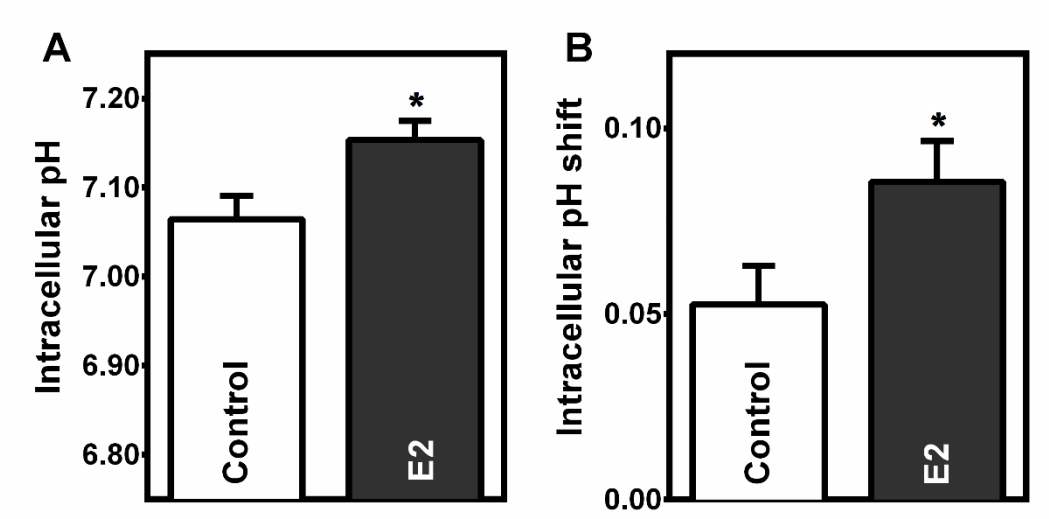

Figure 6.4 Effect of 17β-estradiol (E2) on human Sertoli cells (SCs) intracellular pH. (**A**) represents the pHi in control group and E2-treated SCs. (**B**) shows the pHi shift in both groups (control and E2 group) after stimulation with ATP. Results are expressed as means ± SEM (n=6 for each condition). Significantly different results (T-test, $p< 0.05$) are indicated: * = vs to control group.

E2 is a modulator of ionic transcellular transport in human Sertoli cells

The ionic transcellular transport across the human SC monolayer was evaluated using the Voltage-Clamp technique. A spontaneous transcellular potential difference of 1.5 ± 0.1 mV was generated across the monolayer of cells cultured in the semi-permeable supports. Under a short-circuited current the SCs generated an Isc of 20 ± 2 μA/cm^2.

When stimulated with ATP, the confluent monolayers exhibited an increase on the transcellular transport of electrolytes. The effect of ATP addition on the Isc originated by SCs in our experimental conditions is represented in Figure 6.5a. The ATP added to the adluminal side induced a biphasic response. Initially, it induced an abrupt increase of Isc, which was followed by a consistent decrease (recovery) to a value near the initial Isc (Figure 6.5a).

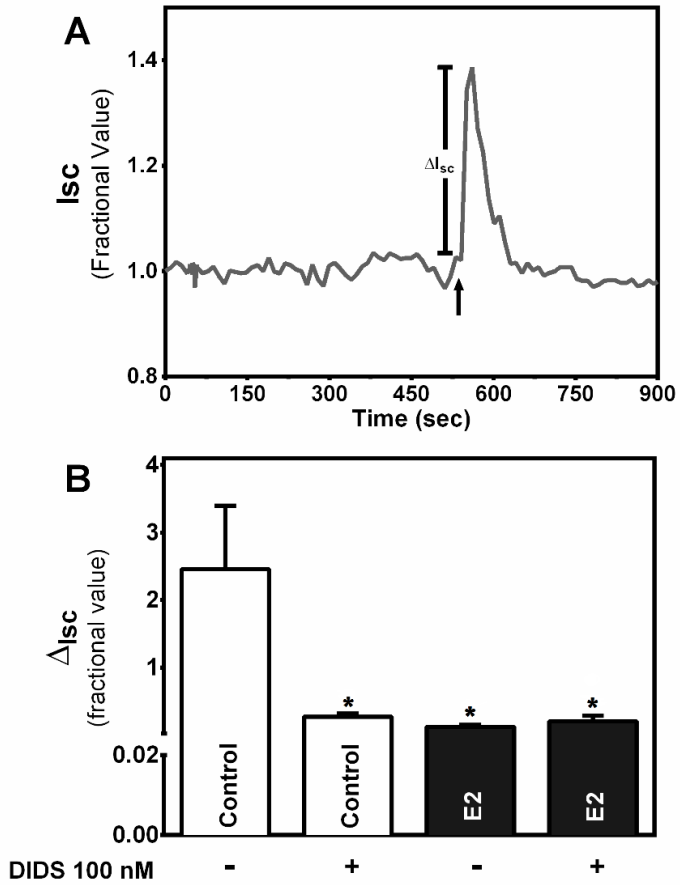

Figure 6.5 Effect of 17β-estradiol (E2) on human Sertoli cells (SCs) transcellular transport. Short-circuit current (Isc) response to the addition of ATP to the adluminal bathing solution. (**A**) shows the representative curve of Isc when ATP was added. (**B**) shows the fractional values of the variation of Isc (ΔIsc) after addition of 2 mM ATP to the adluminal bathing solution in SCs from the control group and E2-treated group. The membrane transport system inhibitor DIDS (0.5 mM) was added to two groups as indicated in Methods section. Results are expressed as means ± SEM (n=6 for each condition). Significantly different results (one-way ANOVA, $p< 0.05$) are indicated: * = vs to control group (-/-). Arrow represents the addition of ATP (2 mM).

When SCs were stimulated by ATP, we observed an increase in Isc (ΔIsc) of 2.5 ± 0.9 units (Figure 6.5b, $P<0.0001$). Notably, when these cells were exposed to the HCO_3^- membrane transport systems inhibitor (DIDS) in the basal side, we observed a smaller ΔIsc of only 0.27 ± 0.05 units as compared with non-exposed cells (Figure 6.5b, $P<0.0001$). We also evaluated the effect of E2 on the transcellular transport by SCs. Exposure to E2 caused a clear perturbation on the effect of ATP in these cells. E2-treated human SCs presented a reduced magnitude of the ATP-stimulated electrogenic transport of electrolytes (ΔIsc). Indeed, we observed that treatment of SCs with E2 caused a decrease of ΔIsc in 0.12 ± 0.03 units as compared to cells of the control group. The exposure to DIDS in the basal side of E2-treated

SCs also led to significant alterations on the shift of ΔIsc promoted by ATP, which was decreased to 0.20 ± 0.08 units (Figure 6.5b, P<0.0001).

Discussion

The presence of diverse HCO_3^- transport systems with distinct expression levels, in testicular cells and spermatozoa, is clear evidence that HCO_3^- plays a central role in the maintenance of male reproductive health. The mechanisms that regulate HCO_3^- concentration throughout the male reproductive tract are essential for determining the ionic composition, osmolarity and pH of luminal fluids [15, 27-29]. Alterations in these processes may end up in male subfertility and/or infertility. In the present study, we identified for the first time the presence of NBCe1, NBCn1 and NDCBE in isolated human SCs. Furthermore, we determined the subcellular localization of these transporters. In isolated hSCs, NDCBE is located in the adluminal region, while NBCn1 and NBCe1 are located in the basal portion of human SCs plasma membrane. Our results provide evidence that NDCBE operates as an acid extruder contributing to pHi regulation by transporting extracellular Na^+ and HCO_3^- from the adluminal compartment in exchange for intracellular Cl^-. Thus, it must play an important role in cellular alkalinisation and possibly on the regulation of the luminal environment. On the other hand, NBCn1 and NBCe1 operate as acid extruders, contributing to pHi regulation and transepithelial acid-base movement. Moreover, these movements are known to occur with a stoichiometry of 1:1 or 1:2 in NBCn1 and NBCe1, respectively (for review, see [16]).

It has been suggested that E2 can regulate the expression of ion transporters involved in pH homeodynamics in the male reproductive tract [12], though the mechanisms remain unknown. Within the testes, SCs are the main targets for hormonal regulation and E2 is known to modulate their physiology (for review, see [16]). Thus, we evaluated the effect of E2 in the expression and functionality of the identified NCBTs. The concentration of E2 was chosen to mimic the maximum concentrations reported for the testicular environment (up to 100 nM) [30]. Our data showed that E2 treatment induced important alterations in the protein levels of NCBTs in human SCs. Indeed, exposure to E2 caused an increase in the protein levels of NDCBE, NBCn1 and NBCe1, suggesting that ion homeodynamics in these cells could be compromised. Therefore, we conducted functional studies to better understand the regulatory mechanisms by which E2 alter HCO_3^- membrane transport systems in human SCs.

Using the H^+-sensitive fluorescent probe BCECF, we determined the impact of E2 on the pHi of human SCs. Notably, human SCs treated with E2 showed an increase in intracellular H^+ concentration. These results are concurrent with the increased protein expression of the three NCBTs in human SCs exposed to E2, as all these proteins transport HCO3- into the cell. Whereas NBCe1 and NBCn1 operate moving HCO_3^- and Na^+ from the basal compartment into

the cell, at the expense of the Na^+ gradient, NDCBE transports luminal HCO_3^- and Na^+ in exchange for intracellular Cl^-, promoting an increase of intracellular Na^+ and HCO_3^- levels within the cell (and hence of pHi) and also a decrease of intracellular content of Cl^-. The nature of the mechanisms implicated in SCs pHi regulation is not fully disclosed yet. As the capacity of SCs to regulate pHi is an important aspect of its physiology, particularly because this cellular parameter may play a major role on the response to hormonal stimulation, as well as on the determination of seminiferous fluid pH, the enlightenment of the contribution of specific membrane transporters to these cells pHi is of great relevance [20]. Thus, we determined the effect of E2 on human SCs pHi after ATP stimulation, which is known to promote an increase in Cl^- secretion dependent on HCO_3^- uptake [26, 31]. While this has not yet been described for SCs, in other cells and tissues, ATP exerts its effects on ion dynamics by direct interactions with ligand-gated ion channels or through G-protein coupled receptors [32]. For instance, it is known that in smooth muscle cells ATP activates ligand-gated ion channel and stimulates Ca^{2+} transport, while in immune cells and osteoblasts ATP stimulates Ionic current by interacting with G-protein-coupled receptors [33].

Our data showed that purinergic stimulation of human SCs leads to an increase of pHi that can be a consequence of the intracellular increase of HCO_3^- via its uptake. Exposure to E2 caused an increase on the pHi shift after ATP stimulation when compared to the control group. These results were consistent with the alterations observed in the expression levels of the studied NCBTs. In this condition, the increased expression of these proteins can lead to higher HCO_3^- uptake into the cell, after ATP stimulation, and the consequent rise of pHi.

To assess the significance of protein and pHi changes induced by the exposure to E2 in human SCs, we conducted functional studies, laying hand of the voltage-clamp technique to unveil the effect of E2 on transcellular transport in these cells. To do so, we performed a similar protocol to the one used by Ko and collaborators [31], which describes that cultured SCs stimulated with ATP increase the electrogenic transport of electrolytes (Isc). This purinergic effect was attributed to an increased HCO_3^--dependent Cl^- secretion [26], resulting in an increase in intracellular HCO_3^- and a consequent increase in pHi. In our study, when human SCs were stimulated with ATP, we also observed an increase on the electrogenic ion transport (ΔIsc). This increase was reduced when DIDS, an inhibitor of HCO_3^- transport systems [34, 35], was placed in contact with the basal portion of the cells' membrane. In these conditions, since NBCe1 and NBCn1 were the only NCBTs identified in the basal portion of polarized human SCs membrane, and NBCn1 has been reported as insensitive to DIDS [35, 36], the exposure to this compound inhibited NBCe1. Hence, the observed effect on ΔIsc when cells were exposed to DIDS was certainly due to the reduction of the HCO_3^- import caused by NBCe1 inhibition. We also evaluated the effect of E2 on the stimulation of Isc by ATP in human

SCs and our data showed that when cells were treated with E2, the magnitude of ΔIsc was also reduced. This decrease of ΔIsc must be due to the alteration of ion homeodynamics observed in these cells after exposure to E2. As referred, human SCs treated with E2 exhibited an increase on the expression of the three NCBTs (NDCBE, NBCn1 and NBCe1), which causes an increase in the influx of HCO_3^- to the intracellular milieu, contributing not only to the increase of pHi but also to the decrease of Cl^- intracellular concentration (due to the augmented expression of NDCBE). As the purinergic effect on Isc was attributed to an increased secretion of Cl- through the adluminal membrane of SCs, by an ATP-activated Cl^- conductance [31], and in E2-exposed human SCs the intracellular Cl^- concentration is decreased, due to augmented expression of NDCBE (which exports this ion into the adluminal compartment), when Isc is stimulated, the secretion of Cl^- will be reduced, thereby leading to reduction of the ΔIsc, as observed. In addition, in the presence of DIDS, we also observed a decrease of polarized human SCs ΔIsc after exposure to E2. In these conditions, the decrease of intracellular Cl^- concentration in response to the overexpression of NDCBE (located at the adluminal membrane) seems to be overlapping with the effect of HCO_3^- import reduction caused by the inhibition of NBCe1. In fact, when we stimulated the Cl^- channels with ATP we observed a reduction of ΔIsc in a similar magnitude as to that observed on cells exposed solely to E2 or to DIDS, which seems to indicate that these two effects are not cumulative.

Taken together, the present results illustrate that E2 is able to modulate the expression and function of the three most significant NCBTs of the SLC4 family in human SCs. Although observations in vitro may not exactly represent an in vivo situation, the obtained results represent a further step in the identification of the key mechanisms by which E2 can regulate SCs physiology and consequently spermatogenesis, with a putative influence in the reproductive capacity of the individuals. In face of the obtained results and the several cases of male infertility related with high E2 levels, it is imperative to further disclose the molecular mechanisms involved in ion transporters expression and regulation in human SCs. Moreover, it is also important to identify and counteract possible alterations associated with pathological conditions that compromise the male reproductive potential, particularly in infertility cases associated with estrogen deregulation.

References

1. Lutz, W., 2006, Fertility rates and future population trends: will Europe's birth rate recover or continue to decline? International Journal of Andrology, 29(1): p. 25-33.
2. Rittenberg, V. and T. El-Toukhy, 2010, Medical treatment of male infertility. Human Fertility, 13(4): p. 208-216.

3. Andersson, A.-M., et al., 2004, Impaired Leydig cell function in infertile men: a study of 357 idiopathic infertile men and 318 proven fertile controls. The Journal of Clinical Endocrinology and Metabolism, 89(7): p. 3161-3167.
4. Pavlovich, C.P., et al., 2001, Evidence of a treatable endocrinopathy in infertile men. Journal of urology, 165(3): p. 837-841.
5. Alves, M.G., et al., 2013, Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cellular and Molecular Life Sciences, 70(5): p. 777-793.
6. Fisher, J., et al., 1997, Immunolocalisation of oestrogen receptor-α within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. Journal of Endocrinology, 153(3): p. 485-495.
7. Hess, R.A., et al., 1997, Estrogen receptor (α and β) expression in the excurrent ducts of the adult male rat reproductive tract. Journal of Andrology, 18(6): p. 602-611.
8. Prossnitz, E.R., et al., 2008, Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annual review of physiology, 70: p. 165-190.
9. Oliveira, P.F., et al., 2014, Expression pattern of G protein-coupled receptor 30 in human seminiferous tubular cells. General and comparative endocrinology, 201: p. 16-20.
10. Carreau, S. and R.A. Hess, 2010, Oestrogens and spermatogenesis. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 365(1546): p. 1517-1535.
11. Joseph, A., B.D. Shur, and R.A. Hess, 2011, Estrogen, efferent ductules, and the epididymis. Biology of Reproduction, 84(2): p. 207-217.
12. Zhou, Q., et al., 2001, Estrogen action and male fertility: roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. Proceedings of the National Academy of Sciences, 98(24): p. 14132-14137.
13. Vaughan-Jones, R.D. and K.W. Spitzer, 2002, Role of bicarbonate in the regulation of intracellular pH in the mammalian ventricular myocyte. Biochemistry and cell biology, 80(5): p. 579-596.
14. Chen, Y., et al., 2000, Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science, 289(5479): p. 625-628.
15. Casey, J.R., S. Grinstein, and J. Orlowski, 2009, Sensors and regulators of intracellular pH. Nature reviews Molecular cell biology, 11(1): p. 50-61.
16. Bernardino, R.L., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current medicinal chemistry, 20(32): p. 4037-4049.
17. Alper, S.L., 2006, Molecular physiology of SLC4 anion exchangers. Experimental physiology, 91(1): p. 153-161.

18. Parker, M.D. and W.F. Boron, 2013, The divergence, actions, roles, and relatives of sodium-coupled bicarbonate transporters. Physiological reviews, 93(2): p. 803-959.
19. Oliveira, P., et al., 2009, Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. Journal of Membrane Biology, 227(1): p. 49-55.
20. Oliveira, P.F., et al., 2009, Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction, 137(2): p. 353-359.
21. Mruk, D.D. and C.Y. Cheng, 2004, Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocrine reviews, 25(5): p. 747-806.
22. Tuck, R., et al., 1970, The composition of fluid collected by micropuncture and catheterization from the seminiferous tubules and rete testis of rats. Pflügers Archiv, 318(3): p. 225-243.
23. Simoes, V., et al., 2013, Regulation of apoptotic signaling pathways by 5α-dihydrotestosterone and 17β-estradiol in immature rat sertoli cells. Journal of Steroid Biochemistry and Molecular Biology, 135: p. 15-23.
24. Petersen, C. and O. Soder, 2005, The sertoli cell--a hormonal target and'super'nurse for germ cells that determines testicular size. Hormone research, 66(4): p. 153-161.
25. Bernardino, R.L., et al., 2013, Effect of Prediabetes on Membrane Bicarbonate Transporters in Testis and Epididymis. The Journal of membrane biology, 246(12): p. 877-883.
26. Ko, W., et al., 1998, Regulated anion secretion in cultured epithelia from Sertoli cells of immature rats. The Journal of physiology, 512(2): p. 471-480.
27. Turner, T., Necessity's potion: inorganic ions and small organic molecules in the epididymal lumen, in The Epididymis: From Molecules to Clinical Practice. 2002, Robaire B and Hinton B.T., Editors. Springer, p: 131-150.
28. Au, C. and P. Wong, 1980, Luminal acidification by the perfused rat cauda epididymidis. The Journal of physiology, 309(1): p. 419-427.
29. Breton, S., et al., 1998, Proton secretion in the male reproductive tract: involvement of Cl−-independent transport. American Journal of Physiology-Cell Physiology, 275(4): p. C1134-C1142.
30. Hess, R.A., 2000, Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Reviews of Reproduction, 5(2): p. 84-92.
31. Ko, W., et al., 1996, Apical ATP stimulates electrogenic anion secretion in cultured Sertoli cells from immature rats. The Journal of physiology, 499: p. 16.
32. Valera, S., et al., 1994, A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. Nature 371(6497): p. 516-519.

33. Burnstock, G. and G.E. Knight, 2004, Cellular distribution and functions of P2 receptor subtypes in different systems. International review of cytology, 240: p. 31-304.
34. Boron, W.F. and E.L. Boulpaep, 1989, The electrogenic Na+/HCO3- cotransporter. Kidney International, 36(3): p. 392-402.
35. Boron, W.F., 2001, Sodium-coupled bicarbonate transporters. Jop, 2(4 Suppl): p. 176-181.
36. Boedtkjer, E., J. Praetorius, and C. Aalkjaer, 2006, NBCn1 (slc4a7) mediates the Na+-dependent bicarbonate transport important for regulation of intracellular pH in mouse vascular smooth muscle cells. Circulation research, 98(4): p. 515-523.
37. Jarow, J.P., et al., 2001, Assessment of the androgen environment within the human testis: minimally invasive method to obtain intratesticular fluid. Journal of andrology, 22(4): p. 640-645.
38. Alves, M.G., et al., 2012, In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1823(8): p. 1389-1394.
39. Oliveira, P., et al., 2012, Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochimica et Biophysica Acta (BBA)-General Subjects, 1820(2): p. 84-89.
40. Skalli, M., et al., 1992, Opposite vectorial secretion of insulin-like growth factor I and its binding proteins by pig Sertoli cells cultured in the bicameral chamber system. Endocrinology, 131(2): p. 985-987.
41. Jesus, T.T., et al., 2014, Aquaporin-9 is expressed in rat Sertoli cells and interacts with the cystic fibrosis transmembrane conductance regulator. IUBMB life, 66(9): p. 639-644.
42. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and cellular biochemistry, 408(1-2): p. 47-54.
43. Thomas, J.A., 1985, Intracellularly trapped pH indicators. Society of General Physiologists series, 40: p. 311-325.
44. Oliveira, P., et al., 2004, H+ ATPase of crude homogenate of the outer mantle epithelium of Anodonta cygnea. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 139(4): p. 425-432.

Chapter 7

A stopped-flow light scattering methodology for assessing the osmotic water permeability of whole Sertoli cells

This chapter was adapted from the published work:

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A stopped-flow light scattering methodology for assessing the osmotic water permeability of whole Sertoli cells

Abstract

Movement of water into and out of the cell is fundamental to life. In the male reproductive tract, at level of the seminiferous epithelium, Sertoli cells are pivotal in mediating the movement of water into the luminal compartment, a process providing a mean of transport of spermatozoa into the epididymal ducts. Here, we describe a stopped-flow light scattering methodology to easily assess the Sertoli cells osmotic water permeability. The devised methodology is well suited for studying how the Sertoli cells permeability changes following exogenous stimuli.

Introduction

Fluid re-absorption and secretion are essential processes in male reproductive tract physiology [1]. Water movements provide a means of transport to the spermatozoa into the epididymal ducts and are therefore essential for composition of the luminal fluids that fill the testicular ducts [2]. In this context, Sertoli cells play a critical role in regulating the amount of water and solutes that move from the interstitial fluid to the adluminal compartment, which is filled by the seminiferous tubular fluid [3]. The solute transporters and water at the Sertoli cells plasma membrane are influenced by various exogenous conditions [4-7]. Sertoli cells express various membrane transport proteins, allowing them to control both the seminiferous fluid composition and pH [2,5,8-10].

Stopped flow light scattering (SFLS) is one of the most widely used approaches in measuring the osmotic and solute permeability of biological membranes. Depending on the solid shape of the cells SFLS can be used to measure the membrane permeability of whole intact cells (cells of spheroidal shape) or that of vesicles prepared from any type of membrane (i.e., plasma membrane, microsomal membranes). SFLS can be also employed to measure the membrane permeability of isolated organelles such as mitochondria [11]. This chapter describes a SFLS-based methodology to measure the coefficient of osmotic water permeability (Pf; cm/s) of the Sertoli cell plasma membrane. The osmotic water permeability is assessed by measuring the cell shrinkage induced by imposing a hyperosmotic gradient (140 mosM) between the extracellular medium and the intracytoplasmic compartment of suspended Sertoli cells. Immediately, after applying the hypertonic gradient, water outflow occurs, and the cells shrink, causing an increase in scattered light intensity [12,13]. The time course of cell volume change is followed from changes in intensity of scattered light at 20 °C (depending on the experimental need, the measurements can be done at lower or higher temperatures) at the wavelength of

530 nm using a stopped-flow reaction analyzer. The Sertoli cells Pf value is calculated using the following equation:

$$P_f = k_i * V_0 / A_v * V_w * \Delta C$$

where ki is the fitted exponential rate constant, V0 is the initial mean cells volume, Av is the mean cells surface, Vw is the molar volume of water, and ΔC is the osmotic gradient [12].

Materials

- **Rat Sertoli cells culture**

The preparation of solutions for rat Sertoli cells culture is done in a sterile environment and ultrapure water should be used to prepare all solutions.

1. Phosphate-buffered Saline Solution (PBS): 140 mM sodium chloride, 3 mM potassium chloride, 10 mM sodium phosphate dibasic and 1.8 mM potassium phosphate monobasic anhydrous. Weigh all reagents into a 1 L graduated cylinder and dissolve in 900 mL of water. If necessary, the pH of the solution should be adjusted to 7.4, using concentrated solutions of hydrochloric acid or/and sodium hydroxide. Adjust the volume to 1 L with deionized water. The PBS should be filtered through a 0.2 µm mixed cellulose ester membrane filter using a vacuum filtration unit in the laminar flow chamber in a sterile environment, and stored in a sterile glass bottle at 4 ºC.

2. Culture Medium: For the preparation of culture medium, should adding Dulbecco's Modified Eagle's Medium - DMEM (with glucose and L-glutamine) with Nutrient Mixture F-12 Ham (with glucose and L-glutamine) and dissolve in 900 mL of water according to the manufacturer's recommendations. After, add 14 mM sodium hydrogen carbonate and 15 mM HEPES. If low-glucose DMEM medium is used, it should be supplemented with D-Glucose until reaching a final concentration of 18 mM. The pH of the medium should be adjusted to 7.4, using concentrated solutions of hydrochloric acid or/and sodium hydroxide. Adjust the volume to 1 L. This solution should be filtered through a 0.2 µm mixed cellulose ester membrane filter using a vacuum filtration unit in the laminar flow chamber in a sterile environment. Add to the sterile medium 50 mL fetal bovine serum (FBS) (10%), 50 µg/mL gentamicin, 50 U/mL penicillin and 50mg/mL streptomycin (all these reagents should be sterile). Store in a sterile bottle at 4 ºC.

3. Trypsin EDTA solution

Stopped-flow Light Scattering

1. Isotonic medium (300 mosM): 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA and 5 mM EGTA, pH 7.4. Store at 4 °C.
2. Hypertonic solution (500 mosM): Weigh 1.822 g of mannitol and transfer to a 50 mL tube containing 50 mL of isotonic medium.
3. Hypotonic solution (220 mosM): Transfer 36.67 mL of isotonic medium to a 50 mL tube and add distilled water until 50 mL.
4. Distilled water
5. Ethanol 70 %
6. Spectrophotometer (Jasco FP-6200)
7. Stopped-Flow injection module
8. Two 10 mL syringes for SFM-20
9. Cryostat

Methods

Here we describe the procedure for measuring the osmotic membrane permeability of Sertoli cells using the equipment already described in the section Materials. The protocol for preparing the Sertoli cell cultures is described in detail in other chapter. In the procedure reported below we used Sertoli cells cultures at about 80% confluence.

Sertoli cell cultures

1. Observe the cells using an inverted optical microscope.
2. Discard the culture medium.
3. Wash the cell culture three times with PBS (4 °C).
4. Trypsinize the cell culture during approximately 5 minutes (see Note 1 and 2).
5. Stop the action of trypsin with FBS-containing culture medium (see Note 3).
6. Centrifuge the cellular suspension at 300xg for 6 min at 4 ºC and discard the related supernatant.

Stopped-flow light scattering setup

1. Before starting the permeability measures, wash the injection system thoroughly (see Note 4).
2. Set the cryostat temperature to 20 °C (see Note 5).
3. Activation of the injection system and the spectrophotometer using the appropriate software.
4. Set SFLS device parameters as follows:
4.1 dead time: 1.6 ms;

4.2 99% mixing efficiency in <1 ms

4.3 mixing time: 20 ms;

4.4 volume injected by syringe 1: 100 µL;

4.5 volume injected by syringe 2: 100 µL.

4.6 Set the excitation and emission wavelength on the stopped-flow spectrophotometer to 450 nm. The bandwidth of excitation and emission must be set to 20 nm.

Sample preparation and stopped-flow light scattering measurements

1. Resuspend the cellular pellet in hypotonic medium and mix by gently pipetting (see Note 6).

2. Measure the mean diameter of the cells using an inverted microscope with camera (see Note 7) (Figure 7.1).

3. Prepare the cellular suspension for one of the syringes of the stopped-flow apparatus diluting 600 µL of the initial cell suspension into 2.5 mL of the hypotonic solution (see Note 8). Let the Sertoli cells equilibrate for 5 min (see Note 9), and then fill the syringe with this cellular suspension.

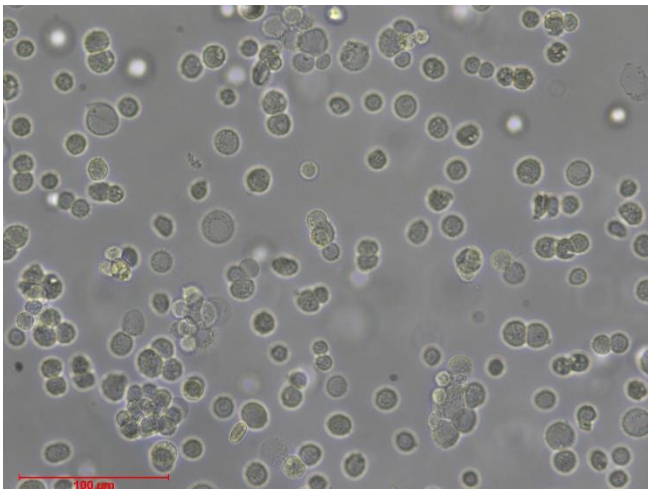

Figure 7.1 Representative image of the rat Sertoli cells suspension. Rat Sertoli cells resuspended and maintained in hypotonic solution (220 mosM) during 5 minutes. Scale bar, 100 µm.

4. Fill the other syringe with the hypertonic solution. (see Note 8)

5. Rapidly mixing in a chamber the hypertonic solution (500 mosM) with the cell suspension (220 mosM) (osmotic gradient: 140 mosM) (Figure 7.2).

6. After applying a hypertonic gradient, water outflow occurs leading to cells shrink and causing an increase in scattered light intensity (I) over time (s).

Results analysis

The fitting of the curve data using a single exponential function allows the determination the related time constant (ki, s^{-1}) of the water.

1. Analyze the kinetics of the fitted curve by placing 3 vertical bars: 1- at the beginning of the exponential phase of the curve; 2 - positioned immediately at the end of the exponential phase of the curve (before at the beginning of the equilibrium phase); 3 - positioned at the end of the equilibrium phase (plateau phase) (Figure 7.3).

2. With these points, the appropriate software (Bio-kine32) automatically calculates the ki of the curve, which is directly related to the slope of the curve.

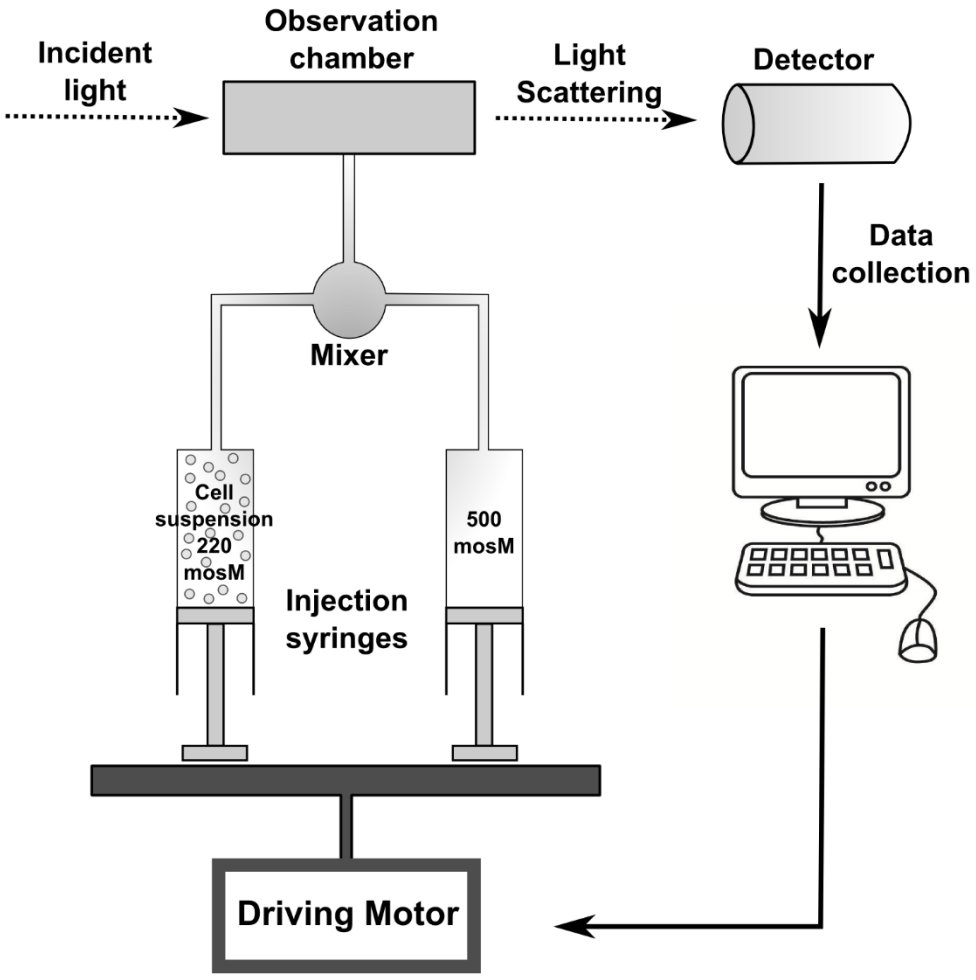

Figure 7.2 Schematic diagram of the stopped-flow light scatering system used for measurment of osmitic permeability of the rat Sertoli cells. A driving motor triggers the syringe-pistons allowing the solutions to move from syringes to a mixer. The mixture passes through an observation chamber, irradiated with light. The detector is connected to the computer in order to collect the data.

3. The ki value is used for calculation of water permeability as described in the Introduction section.

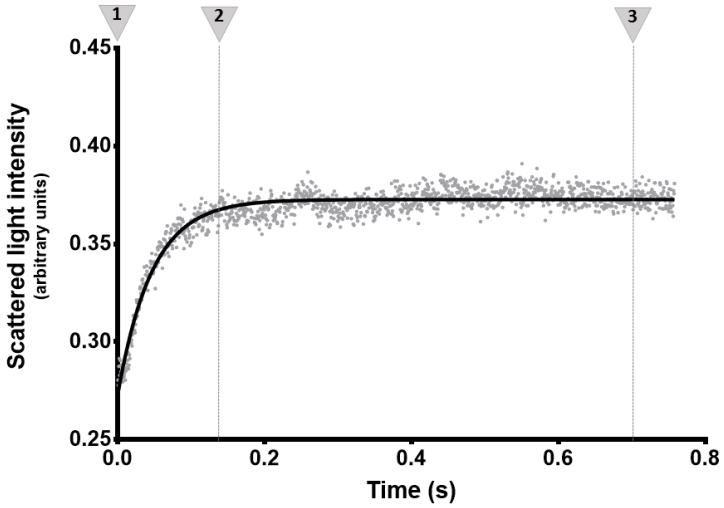

Figure 7.3 Representative trace of a time course of scattered light intensity along with single exponential fits of Sertoli cells exposed to a 140 mOsm hyperosmotic gradient. Lines 1 and 2 represent the start and the end of the exponential phase, respectively. Line 3 is the end of the equilibrium phase.

Notes

1. The volume of trypsin needs to be adapted to the size of the flask; it should cover the entire surface of the flask.

2. The time of incubation with trypsin should be sufficient to obtain a cellular suspension of individualized cells.

3. The quantity FBS-containing culture medium should be equal to the volume of trypsin.

4. For both syringe housings, perform three washings with distilled water, 70% ethanol and distilled water, respectively. Repeat these steps at the end of the procedures.

5. It is advisable to start the measurements once the device reaches the selected temperature. Measurements can be done at different temperatures although for the procedure described in this chapter we worked at 20 °C.

6. The cells are equilibrated in a slightly hypotonic solution to increase cell turgidity. The volume of the hypotonic solution should be adjusted to the number of the cells. In average one T75 flask with 80% confluence contains about $1x10^7$ Sertoli cells (e.g., the pellet obtained the six T75 flasks with 80% confluence should be diluted in approximately 2 mL of hypotonic solution).

7. Capture the images with a microscope equipped with a camera and measure the cell diameter with an appropriate software.
8. Prepare fresh hypotonic and hypertonic solutions before doing experiment.
9. Equilibration of Sertoli cells in the 220 mosM solution and osmotic shock 500 mosM (equal volumes of cell suspension and hypertonic solution upon mixing) leads to an outwardly directed osmotic gradient of 140 mosM.

References

1. Martins, A.D., et al., 2014, Physiology of Na+/H+ Exchangers in the Male Reproductive Tract: Relevance for Male Fertility Biology of Reproduction, 91(1): p. 1-6.
2. Rato, L., et al., 2010, Tubular fluid secretion in the seminiferous epithelium: ion transporters and aquaporins in Sertoli cells. Journal of Membrane Biology, 236(2): p. 215-224.
3. Bernardino, R., et al., 2013, Molecular basis of bicarbonate membrane transport in the male reproductive tract. Current Medicinal Chemistry, 20(32): p. 4037-4049.
4. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and Cellular Biochemistry, 408(1-2): p. 47-54.
5. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.
6. Jesus, T.T., et al., 2014, Aquaporin-4 as a molecular partner of cystic fibrosis transmembrane conductance regulator in rat Sertoli cells. Biochemical and Biophysical Research Communications, 446(4): p. 1017-1021.
7. Jesus, T.T., et al., 2014, Aquaporin-9 is expressed in rat Sertoli cells and interacts with the cystic fibrosis transmembrane conductance regulator. IUBMB Life, 66(9): p. 639-644.
8. Bernardino, R.L., et al., 2016, Hepatocyte and Sertoli Cell Aquaporins, Recent Advances and Research Trends. International Journal of Molecular Sciences, 17(7): p. E1096.
9. Oliveira, P.F., et al., 2009, Membrane transporters and cytoplasmatic pH regulation on bovine Sertoli cells. The Journal of membrane biology, 227(1): p. 49-55.
10. Oliveira, P.F., et al., 2009, Intracellular pH regulation in human Sertoli cells: role of membrane transporters. Reproduction, 137(2): p. 353-359.
11. Mathai, J.C., G.D. Sprott, and M.L. Zeidel, 2001, Molecular mechanisms of water and solute transport across archaebacterial lipid membranes. Journal of Biological Chemistry, 276(29): p. 27266-27271.
12. Calamita, G., et al., 2008, Altered expression and distribution of aquaporin-9 in the liver of rat with obstructive extrahepatic cholestasis. American Journal of Physiology-Gastrointestinal and Liver Physiology, 295(4): p. G682-G690.

13. Calamita, G., et al., 2005, The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water. Journal of Biological Chemistry, 280(17): p. 17149-17153.

Chapter 8

Estrogen modulates glycerol permeability in Sertoli cells through downregulation of Aquaporin-9

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Abstract

High 17β-Estradiol (E2) levels are known to cause alterations on spermatogenesis and environments throughout the male reproductive tract. Sertoli cells (SCs) ensure an adequate environment inside the seminiferous tubule. Glycerol stands as essential for the maintenance of blood-testis barrier created by SCs, however the role of E2 in this process is not known. Herein, we hypothesized that the effect of E2 on glycerol permeability in mouse SCs (mSCs) could be mediated by aquaglyceroporins. The expression of aquaglyceroporins was performed by RT-PCR and qRT-PCR. Glycerol permeability was evaluated by stopped-flow light scattering. We were able to identify the expression of AQP3 and AQP9, in mSCs where AQP9 is more abundant than AQP3. Our results show that high E2 levels decrease AQP9 mRNA abundance with no influence on AQP3 in mSCs. Interestingly, high E2 levels decreased mSCs permeability to glycerol, while downregulating AQP9 expression, thus suggesting a novel mechanism by which E2 modulates fluid secretion in the testis. In conclusion, E2 is an important regulator of mSCs physiology and secretion through changes in AQP9 expression and function. Thus, alterations in glycerol permeability induced by E2 may be the cause for male infertility in cases associated with the presence of high E2 levels.

Keywords: Aquaglyceroporins, Aquaporin 9, Estrogen, Glycerol, Sertoli cells.

Introduction

In the last two decades, extensive research showed that beyond the classic view on the major relevance of androgens for male reproduction, estrogens can also regulate the development and function of the male reproductive tract. 17β-Estradiol (E2) is found in measurable concentrations in the blood of men and higher concentrations have been reported in the testis and semen [1, 2]. E2 is necessary for the healthy development and function of the male reproductive organs. However, elevated levels of this hormone induce deleterious effects that are consistently associated with male subfertility or infertility [2, 3]. For instance, elevated E2 levels induce morphological alterations in SCs and Leydig cells that end-up in severe failures in spermatogenesis [4].

High E2 levels change ionic homeostasis and the transport of some molecules within the seminiferous tubule, being that some of those molecules may alter or even arrest

spermatogenesis. For instance, elevated concentrations of glycerol in testis causes changes in the function of the blood-testis barrier (BTB), compromising the homeostasis of the tubular fluid and leading to the death of germ cells [5]. In fact, while glycerol is essential for spermatogenesis [6], acute exposition to high concentrations of glycerol can temporarily halt spermatogenesis [7]. In addition, chronic exposition to high glycerol concentrations may prompt permanent oligospermia or even azoospermia [5]. SCs are responsible for the maintenance of the BTB [8] and thus, for promoting a normal spermatogenesis. However, studies concerning SCs permeability to glycerol and the expression and function of the associated transport mechanisms are still scarce.

Aquaporins (AQPs) are a family of channel proteins facilitating the transport of water and a series of small anaelectrolytes across biological membranes [9]. Aquaglyceroporins represent a subgroup of the AQPs family conducting not only water but also small neutral solutes, such as glycerol [10]. Mammalian aquaglyceroporins comprise four known homologues – AQP3, AQP7, AQP9, and AQP10. AQPs are widely located in the male reproductive tract, where they are involved in fluid absorption/secretion dynamics and contribute in maintaining the homeostasis for the occurrence of a normal spermatogenesis [9, 11]. Hence, AQPs have emerged as pivotal for healthy male reproductive function and alterations in their expression or function have been suggested to result in subfertility or infertility. Furthermore, there is evidence that estrogens regulate the expression and function of AQPs in efferent ducts and epididymis. It was reported that estrogens modulate water reabsorption through AQP9, which may compromise sperm quality [12, 13]. These works led us to envisage that male infertility may be linked to elevated E2 levels and altered expression or function of aquaglyceroporins associated with glycerol permeability in SCs.

Herein, we hypothesized that E2 may regulate glycerol permeability through aquaglyceroporins expression and function in mouse testis, particularly in mouse SCs (mSCs). Thus, the aim of this study was to evaluate the impact of high concentrations of E2 on aquaglyceroporins expression in mSCs, with focus on the expression of AQP3, AQP7 and AQP9 (the *Aqp10* gene is a pseudogene in mouse) [14]. Since these aquaglyceroporins are responsible for the transport of glycerol whose homeostasis is critical for proper male reproductive health, we further evaluated the impact of high E2 on mSCs glycerol permeability.

Material and Methods

Chemicals

NZY Total RNA Isolation kit and NZY M-MuLV Reverse Transcriptase was acquired from NZYtech (Lisboa, Portugal), Fetal bovine serum from Biochrom AG (Berlin, Germany and all other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise.

Cell culture and experimental groups

Mouse SCs (mSCs), TM4 were purchased from ATCC (Virginia, USA). Cells were seeded in 75 cm^2 flask (SPL70075, SPL Life Sciences, Korea) in 1:1 mixture of DMEM:F12 supplemented with 1.2 g/L sodium bicarbonate, 50 U/mL penicillin, 50 mg/mL streptomycin sulphate, 0,5 mg/mL fungizone, 50 µg/mL gentamicin and 5% FBS with physiological concentrations of testosterone (5 µM) and E2 (1 nM).

Cells were grown until reaching a confluence of 70%–80%. Then the culture medium was replaced by phenol-red free DMEM:F12 medium supplemented with ITS (in mg/L: insulin 10, transferrin 5.5, selenium 0.0067; pH 7.4). Cells were separated in two groups, control group and a group treated with high concentration of E2 (100 nM). The E2 concentration was chosen based on published papers, which demonstrated that in intratesticular plasm levels of this hormone are particularly higher than those of circulating plasma, reaching concentrations up to 200 nM [15, 16]. The same amount of ethanol (solvent) was used in the cells of the control group that was used in the cells of the E2-treated group (<0.025% v/v). Treatments were done during 24 hours in an atmosphere of 6% CO_2 and 94% O_2 at 33ºC.

Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qPCR)

Extraction of RNA from mSCs and mouse testis was performed using the NZY Total RNA Isolation kit as indicated by the manufacturer. RNA concentrations were determined by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, California, USA). RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase. The resultant complementary DNA (cDNA) was used with exon-exon spanning primer sets designed to amplify cDNA fragments described in Table 1.

Conventional reverse transcriptase PCR (RT-PCR) was performed to identify Aqp3, Aqp7 and Aqp9 mRNAs in mSCs and quantitative Real-Time PCR (qPCR) was performed to evaluate the mRNA abundance in cells from the control and E2-treated group as previously described [3]. Briefly, specific primers were designed for the amplification of the Aqp3, Aqp7, Aqp9 and

β-2-microglobulin transcripts. qPCR conditions were previously optimized and specificity of the amplicons was determined by melting curves. Amplification conditions: 5 min at 95 °C, followed by 30 or 40 runs of a 3 steps cycle: 10 s at 95 °C; 30 s with a specific temperature for each set of primers, and 10 s at 72 °C. β2-microglobulin transcript levels were used to normalize gene expression levels. Fold variation of gene expression levels was calculated following the model proposed by Pfaffl [17], using the formula $2^{-\Delta\Delta Ct}$.

Table 8.1 Oligonucleotides and cycling conditions for PCR amplification of Aquaporin-3 (Aqp3), Aquaporin-7 (Aqp7), Aquaporin-9 (Aqp9) and β-2-microglobulin. C: Number of cycles.

Gene	Sequence 5'- 3'	Annealing Tº	C
Aqp3 (NM_016689.2)	**FWD:** GGACCCTCATCCTTGTGATGTT **RVS:** TCGTAGTACAGCCCAAAAACAA	63ºC	40
Aqp7 (NM_007473.4)	**FWD:** CTACAGAAGAATATGGTGCGAGA **RVS:** CAGGAACTGACCCAGCACAT	63ºC	40
Aqp9 (NM_022026.3)	**FWD:** CTGAGAAGGACCGAGCCAAG **RVS:** ATGATGACGCTGAGTTCGTGT	60ºC	40
β-2-microglobulin (NM_009735.3)	**FWD:** GCTTCAGTCGTCAGCATGGC **RVS:** GGATTTCAATGTGAGGCGGGT	58ºC	30

Preparation of cellular suspension

mSCs obtained from the control and E2-treated groups were detached with trypsin and centrifuged at 300.*g* to obtain a cellular pellet. The cells were resuspended in isotonic medium (300 mOsm, in mM: 220 mannitol, 70 sucrose, 20 Tris-HCl, 1 EDTA, 5 EGTA, 1 PMSF, pH 7.4) and left 10 min to reach the equilibrium in this medium. The cellular preparations were homogeneous and mSCs were spherical in shape when in suspension, as observed under light microscopy. The diameter of cells was measured for all the preparations with ImageJ software with pictures obtained by light microscopy.

Stopped-flow light scattering

Stopped-flow light scattering was performed following an adaptation of the protocol described by Maggio et al. [18] and Campos et al. [19]. Experiments were performed on a HI-TECH Scientific PQ/SF-53 stopped-flow apparatus, which has a 2 ms dead time and is temperature controlled (24ºC), interfaced with an IBM PC/AT compatible 80386 microcomputer. This

procedure was performed to measure the membrane permeability of mSCs to glycerol. Osmotic shock was performed with glycerol solution (540 mOsm, in mM: 150 glycerol, 220 mannitol, 70 sucrose, 20 Tris-HCl, 1 EDTA, 5 EGTA, 1 PMSF, pH 7.4). Four runs were usually stored and analysed in each experimental condition. In each run 0.1 ml cellular suspension was mixed with an equal amount of hyperosmotic glycerol solution to reach inwardly directed gradients of solute. After the first fast cell shrinkage due to water outflow, glycerol influx in response to its chemical gradient was followed by water influx with subsequent cell re-swelling. The kinetics of cell re-swelling was measured from the time course of 90° scattered light intensity at 530 nm until a stable light scatter signal was attained. Glycerol permeability (P_{gly}) was calculated as $P_{gly} = ki(V_0/A)$, where ki is the single exponential time constant (s^{-1}) fitted to the light scattering signal of glycerol influx and V_0/A is the initial cell volume to area ratio.
Glycerol permeability was measured in mSCs control and E2-treated groups. In addition, both groups were incubated with phloretin, a general inhibitor of aquaglyceroporins [20, 21], Phloretin (0.7 mM) was added to the cellular suspension 15 min before permeability measurements, according to previously published data [20, 21]. All solution osmolarities were determined from freezing point depression on a semi-micro osmometer (Knauer GmbH, Berlin, Germany) using standards of 100 and 400 mOsm.

Statistical Analysis

Experimental results are presented as mean ± standard error of mean (SEM) (n=6 for each condition, done in triplicate). Statistical analysis was executed using a one-way ANOVA in GraphPad Prism 6 (GraphPad Software, San Diego, USA). $P<0.05$ was considered significantly different.

Results

Aqp3 and *Aqp9* are expressed in mSCs but not *AQP7*

To date, studies on the role and location of aquaglyceroporins in SCs are virtually inexistent. In this study, we performed a screen to identify the expression of aquaglyceroporins in mSCs. We were able to identify the expression of *Aqp3* and *Aqp9,* but the presence of *Aqp7* was not detected in mSCs (Figure 8.1A). The positive control for *Aqp3* and *Aqp7* was mouse kidney and for *Aqp9* was mouse liver. We did not check the fourth aquaglyceroporin, AQP10, since its gene is known to be a pseudogene in mouse [14].
The relative abundance of the *Aqp3* and *Aqp9* transcripts was determined by qRT-PCR, with the primers efficiency being set to 90% - 110%. In mSCs, the relative abundance of *Aqp9*

(0.0200 ± 0.0029 arbitrary units) resulted about seven times higher than that of *Aqp3* (0.0034 ± 0.0004 arbitrary units) in mSCs (Figure 8.1B).

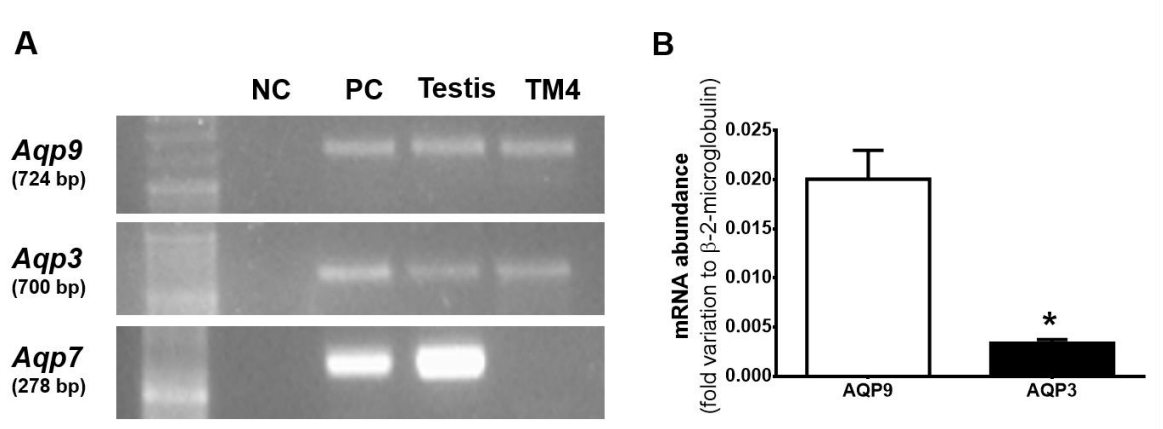

Figure 8.1 Identification and quantification of mRNA abundance of Aquaporin-3 (*Aqp3*), *Aqp7* and *Aqp9* in mouse testis and mouse Sertoli cells (mSCs). **A)** Representative reverse transcriptase-PCR experiment. Mouse liver cDNA was used as positive control for the evaluation of mRNA expression of *Aqp9* and kidney cDNA for *Aqp3* and *Aqp7*. **B)** Relative abundance of *Aqp9* and *Aqp3* mRNA in mSCs. PC: Positive control; NC: Negative control. Results are expressed as mean ± SEM (n = 6). * $p<0.05$

High levels of E2 downregulate *Aqp9* mRNA expression in mSCs

We evaluated the mRNA expression of *Aqp3* and *Aqp9* in mSCs treated with high levels of E2 relatively to cells from the control group. The mRNA abundance of *Aqp3* was not altered in cells treated with E2 (1.34 ± 0.24-fold variation to control) in relation to control group (1.00 ± 0.11-fold variation to control). Concerning *Aqp9* mRNA abundance, we observed a significant decrease when mSCs were treated with high E2 levels (0.29 ± 0.08-fold variation to control) in relation to the expression detected in cells from the control group (1.00 ± 0.28-fold variation to control) (Figure 8.2).

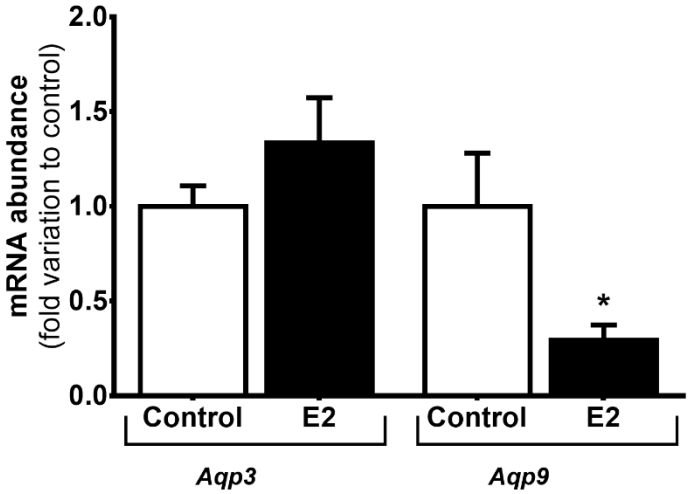

Figure 8.2 Effect of exposure of mouse Sertoli cells (mSCs) to 17β-estradiol (E2) (100 nM) on *Aquaporin-3* (*Aqp3*) and *Aqp9* mRNA abundance. Results are expressed as mean ± SEM (n = 6 for each condition). Results are expressed as mean ± SEM (n = 6). * $p<0.05$ relative to control.

Permeability of mSCs to glycerol is decreased after exposure to high levels of E2

Since aquaglyceroporins allow facilitated diffusion of glycerol across membranes we ran stopped-flow light scattering experiments to evaluate the possible correlation between the expression of the aquaglyceroporins in question and the mSCs membrane permeability. Glycerol permeability (P_{gly}, μm/s) was calculated by evaluating the diminution in light scattering intensity associated to the osmotic influx of water that followed the entry of glycerol into the mSCs triggered by its inwardly-directed chemical gradient (150 mM; see *Materials and Methods* for details). The diameter of mSCs needed to calculate the P_{gly} was measured by optic microscopy before the beginning of the stopped-flow light scattering measurements, when the suspended cells were in osmotic equilibrium with the 300 mOsm solution. The mean cell diameter was 21.50 ± 0.32 μm. Scattered light intensity representative curves of mSCs from control group (A), group exposed to the aquaglyceroporin inhibitor, phloretin (C), E2-treated group (B) and E2-treated group exposed to phloretin (D) are illustrated in Figure 8.3.

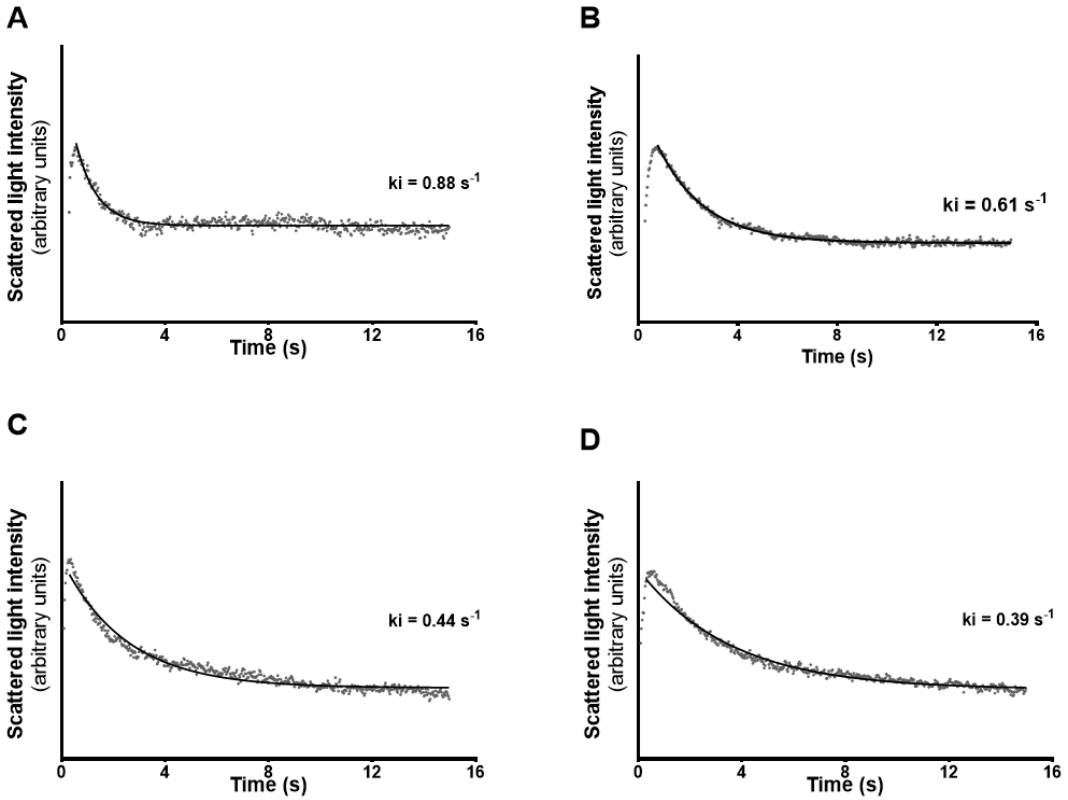

Figure 8.3 Representative curves of scattering light intensity for glycerol permeability in mouse Sertoli cells (mSCs) under different conditions. **A)** Cells from control group, ki= 0.88 s^{-1}; **B)** Cells from group treated with 17β-estradiol (E2) 100 nM, ki= 0.61 s^{-1}; **C)** Cells exposed to phloretin (0.7 mM) ki= 0.44 s^{-1}; **D)** Cells from group treated with 17β-estradiol (E2) 100 nM and after were exposed to phloretin (0.7 mM), ki= 0.39 s^{-1}.

The inhibition of aquaglyceroporins by phloretin reduced the permeability of mSCs to glycerol by 55% in relation to control (44.92 ± 2.29 %, 100 ± 13.38 %, respectively). Additionally, exposure to high levels of E2 also significantly reduced the mSCs permeability to glycerol, likewise cells treated with E2 and exposed to phloretin (54.21 ± 1.97 %, 43.04 ± 1.56 %, respectively) (Figure 8.4). Consistent with the above described aquaglyceroporin downregulation induced by E2 no differences were observed concerning the P_{gly} values in mSCs inhibited with phloretin and those exposed with E2 and then treated with phloretin before the glycerol permeability measurements (Figure 8.4).

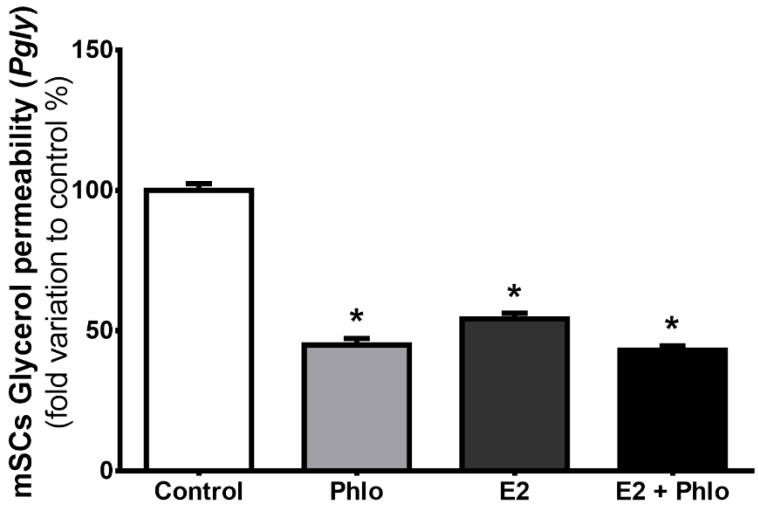

Figure 8.4 Effects of 0.7 mM phloretin, 17β-estradiol (E2) 100 nM and E2 with exposure to phloretin in mouse Sertoli cells permeability to glycerol (P_{gly}). Results are expressed as mean ± SEM (n = 6). *p<0.05 relative to control.

Discussion

The role of estrogens in male reproductive function has been exhaustively investigated in the last decades providing evidence that estrogens are crucial to the establishment of the male reproductive potential [3, 23, 24]. Fluid reabsorption and ion transport in some regions of the male reproductive tract are known to be regulated by steroid hormones [25-28]. Indeed, as also reported in liver [29], steroid hormones, namely E2, can modulate the expression of some aquaglyceroporin isoforms and consequently change the fluid reabsorption in efferent ducts and epididymis [23]. For instance, E2 is able to modulate the transcriptional expression of AQP9 in efferent ducts and epididymis. This effect can occur early during the sexual development, as exposure of neonatal rats to estrogenic compounds decrease the transcript level of AQP9 in epididymis [13], but these effects were reversed with the administration of testosterone [12]. Contrastingly, anti-estrogens cause a down-regulation of AQP9 in efferent ducts [30], which may lead to impaired reabsorption in the seminal fluid. Additionally, in the epididymis, it has been reported that orchiectomized rats were devoid of AQP3 in basal epididymal cells. However, when testosterone was administrated, a slightly restoration of AQP3 expression was noted, suggesting that sex steroid hormones can modulate the expression of AQP3 in the epididymis [31]. In fact, similar effects have also been reported for AQP9 expression [25]. Nevertheless, there is contradictory data regarding this subject. There are studies showing that estrogens do not alter AQP9 expression, while androgens seem to modulate AQP9 levels in the initial segment of the epididymis [29], and other where estrogen

administration increased AQP9 expression in efferent ducts [13]. These inconsistencies in the literature illustrate the complexity surrounding the regulation of aquaglyceroporins. While there is evidence to support that aquaglyceroporins are under sex steroid hormone regulation, few data are available and further research on the topic is required. Moreover, the expression pattern of aquaglyceroporins throughout the male reproductive tract remains mostly unknown. Thus, we hypothesized that E2 could control the expression of plasma membrane aquaglyceroporins and, consequently, the related cell membrane permeability to glycerol in mSCs, as it happens in other cells and regions of the male reproductive tract.

In this work, we were able to identify, for the first time, the expression of three different aquaglyceroporins transcripts in mouse testis - *Aqp3*, *Aqp7* and *Aqp9*. Interestingly, the screening in mSCs showed that *Aqp7* is not expressed, but we found presence of the transcripts of two homologues, AQP3 and AQP9, featuring high permeability to glycerol (in addition to other solutes and water). In addition to these aquaglyceroporins, AQP10 and AQP11 are also able to transport glycerol [32, 33]. AQP10 is present in the efferent ducts and epididymis in rats [31]. However, AQP10 is a pseudogene in mice and does not encode a functional protein [14]. With regard to AQP11, this transporter was identified in the efferent ducts, epididymis, and testes of rats [34]. In testes, AQP11 is expressed in the elongated spermatids and in the residual bodies present in the cytoplasm SCs, being linked with intracellular water and glycerol transport, and regulating organelles volume, and not in the plasma membrane [35]. Being so, as we aimed to evaluate impact of E2 on the expression of cell membrane aquaglyceroporins and on its permeability to glycerol in mSCs, we did evaluate eventual contribution of the three different aquaglyceroporins *Aqp3*, *Aqp7* and *Aqp9*, while not that of AQP10 and AQP11. There are studies showing that glycerol is essential for regular spermatogenesis and for normal testis morphology [6]. Conversely, intratesticular injection of glycerol in rats resulted in long-term suppression of spermatogenesis and increased permeability in the blood-testis barrier (BTB) [36]. As BTB is formed by SCs and glycerol induced alterations in the tight junction formed by the union of SCs in [5], these "nurse cells" appear to be susceptible to the action of this metabolite and may mediate its negative effect in spermatogenesis. Hence, it is of extreme importance to understand the regulatory mechanisms and dynamics of glycerol permeability since it can define the reproductive potential of males by modulating SCs function. Herein, we studied the mechanisms by which mSCs permeability to glycerol occurs and how E2 may modulate it.

Aquaglyceroporins are responsible for the facilitated transport of glycerol across the membranes of the vast majority cells. Our results showed that mSCs express two isoforms of aquaglyceroporins, *Aqp3* and *Aqp9*. In addition, we observed that *Aqp9* is about seven times more expressed transcriptionally in mSCs than *Aqp3*, indicating that *Aqp9* may have a more

relevant role on glycerol and water transport than *Aqp3* in these cells. Permeability studies by stopped-flow light scattering also demonstrated that mSCs permeability to glycerol is inhibited by phloretin in 55% in relation to non-treated cells. Since phloretin is known as a general inhibitor of aquaglyceroporins [22, 37, 38], and that only two isoforms able to transport glycerol are expressed in mSCs, it is clear that AQP3 and AQP9 are pivotal for the transport of glycerol in the mSCs. Glycerol movement across membranes also occur through the phospholipid bilayer, by “simple diffusion”, a thermodynamically disadvantaged pathway, of less importance and, above all, not controllable pathway with respect to the “facilitated” pathway offered by aquaglyceroporins [20, 21].

As referred, previous works have shown that E2 is a regulator of aquaglyceroporins in the male reproductive tract [12, 30]. Indeed, as it happens in the epididymis and efferent ducts, aquaglyceroporins are also modulated by E2 in mSCs. However, there was a differential effect concerning the effects on *Aqp3* or *Aqp9*, since high E2 levels downregulated *Aqp9* expression but did not influence *Aqp3* expression. The downregulation of *Aqp9* expression induced by high E2 levels is consistent with the modulation detected in terms of glycerol transport. Through studies using stopped-flow light scattering technique to assess glycerol permeability in mSCs, we detected a 46% reduction in glycerol permeability after exposure to high levels of E2 in relation to mSCs cultured with physiological concentrations of this hormone. In addition, the inhibition of aquaglyceroporins by phloretin in cells previously treated with high levels of E2 did not cause a significant alteration in glycerol permeability when compared with cells only treated with high levels of E2. Hence, these data suggest that the effect caused by the downregulation of *Aqp9* induced by high levels of E2 is comparable to the inhibitory effect conferred by phloretin on the permeability to glycerol in mSCs.

Our results show that exposure of mSCs to high levels of E2 causes a decrease in *Aqp9* expression, which leads to decreased glycerol permeability and can lead to dysregulation of the concentration of glycerol in the testis causing fertility problems or even infertility. Moreover, the number of cases idiopathic infertility associated with increased levels of E2 and high glycerol levels is high [39] and thus, dysfunction of glycerol transport may play a role in those cases. In addition to cases of idiopathic infertility that account for about 30-45% infertility cases due to male factor [40], there are also some known pathologies that are related to hormonal changes in E2 levels and induce male infertility. For instance, Klinefelter syndrome is the most frequent sex chromosome abnormality in men. Men with this syndrome present in most cases low levels of testosterone and increased levels of estrogens and follicle stimulating hormone (FSH) [3]. Most patients affected by this syndrome present azoospermia with consequent infertility [40]. It may be relevant to assess the expression of aquaglyceroporins in SCs and related glycerol permeability in men with Klinefelter's syndrome. Other disorders, such as

obesity, have a great incidence in the world and are steadily increasing. Obesity is regularly associated with hormonal dysregulation [41, 42]. In addition, obesity is related with alterations in the expression and function of the aquaglyceroporins and consequently in glycerol transport [43, 44]. Obese men are known to have a positive correlation with abnormal sperm morphology, decreased sperm concentration and sperm motility, decreased serum testosterone and increased E2 levels compared with health men, which consequently leads to compromised fertility [45-47]. In male reproductive tract, SCs are the principal hormonal targets [48], and since E2 plays such an important role in male reproduction, namely in glycerol permeability by the control of aquaglyceroporins, the study of this cell type is of great importance also to understand the mechanisms by which overweight and metabolic diseases affect the fertility of males.

However, *in vitro* effects are not always reflected *in vivo*. Although it is known that glycerol is essential for spermatogenesis, no changes were observed in the reproductive parameters of *Aqp9* null mice [49]. Compensatory effects may be occurring *in vivo* in this animal model, or the expression/localization of aquaglyceroporins in SCs *in vivo* may not be exactly the same as in cell cultures. For instance, as in rat testis there is an elevated expression of AQP9 in Leydig cells [50] and these cells strongly influence SCs (namely by the secretion of testosterone), there will be indirect effects on SCs that may be responsible for the reproductive phenotype of *Aqp9* null mice. This is in fact an initial *in vitro* study, which demonstrates that pure cultures of mSCs exposed to high doses of E2 present a decrease in the abundance of *Aqp9* and consequently decrease the permeability to glycerol. *In vivo* studies evaluating the expression and function of aquaglyceroporins in the testicular tissue of individuals with sex steroid hormones dysregulation (particularly estrogens) will be useful to clarify this matter. In summary, we described, for the first time, the expression of two important glycerol channels in mSCs, AQP3 and AQP9, which may be essential for spermatogenesis due to their ability to mediate the membrane transport of glycerol, a metabolite known to arrest germ cell development. E2 is an essential hormone in male reproductive tract and we have now reported a new mechanism by which it may impact male fertility: through downregulation of *Aqp9* modulating SCs glycerol permeability. This study, may open new insight on the cases of male infertility related to increased E2 levels, which may be associated with an alteration of testicular glycerol levels, through changes in *Aqp9* expression, and a consequent negative impact on spermatogenesis. Still, further studies will be needed to correlate these results with an *in vivo* situation and to highlight a possible association between these mechanisms and idiopathic infertility and other diagnosed cases of male infertility associated with high E2 levels.

References

1. Clarke, M. and C.A. Pearl, 2014, Alterations in the estrogen environment of the testis contribute to declining sperm production in aging rats. Systems Biology in Reproductive Medicine, 60(2): p. 89-97.
2. Hampl, R., et al., 2013, Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors. Hormone Molecular Biology and Clinical Investigation, 13(1): p. 1-5.
3. Bernardino, R., et al., 2016, Expression of estrogen receptors alpha (ER-α), beta (ER-β), and G protein-coupled receptor 30 (GPR30) in testicular tissue of men with Klinefelter syndrome. Hormone and Metabolic Research, 48(06): p. 413-415.
4. Leavy, M., et al., 2017, Effects of Elevated beta-Estradiol Levels on the Functional Morphology of the Testis - New Insights. Scientific Reports, 7: p. 39931.
5. Wiebe, J.P., et al., 2000, Glycerol Disrupts Tight Junction—Associated Actin Microfilaments, Occludin, and Microtubules in Sertoli Cells. Journal of andrology, 21(5): p. 625-635.
6. Crisóstomo, L., et al., 2017, Glycerol and testicular activity: the good, the bad and the ugly. Molecular Human Reproduction, 23(11): p. 725-737.
7. Wiebe, J.P., K.J. Barr, and K.D. Buckingham, 1989, Sustained azoospermia in squirrel monkey, Saimiri sciureus, resulting from a single intratesticular glycerol injection. Contraception, 39(4): p. 447-57.
8. Crisostomo, L., et al., 2018, Molecular Mechanisms and Signaling Pathways Involved in the Nutritional Support of Spermatogenesis by Sertoli Cells. Methods in Molecular Biology, 1748: p. 129-155.
9. Bernardino, R.L., et al., 2016, Hepatocyte and Sertoli Cell Aquaporins, Recent Advances and Research Trends. International Journal of Molecular Sciences, 17(7): p. E1096.
10. Sales, A.D., et al., 2013, Structure, function, and localization of aquaporins: their possible implications on gamete cryopreservation. Genetics and Molecular Research, 12(4): p. 6718-32.
11. Yeste, M., et al., 2017, Aquaporins in the male reproductive tract and sperm: Functional implications and cryobiology. Reproduction in Domestic Animals, 52 Suppl 4: p. 12-27.
12. Pastor-Soler, N.M., et al., 2010, Aquaporin 9 expression in the developing rat epididymis is modulated by steroid hormones. Reproduction, 139(3): p. 613-621.
13. Wellejus, A., et al., 2008, Expression of aquaporin 9 in rat liver and efferent ducts of the male reproductive system after neonatal diethylstilbestrol exposure. The Journal of Histochemistry and Cytochemistry, 56(5): p. 425-432.

14. Hess, R.A., 2000, Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Reviews of Reproduction, 5(2): p. 84-92.
15. Jarow, J.P., et al., 2001, Assessment of the androgen environment within the human testis: minimally invasive method to obtain intratesticular fluid. Journal of andrology, 22(4): p. 640-645.
16. Pfaffl, M.W., 2001, A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research, 29(9): p. e45-e45.
17. Maggio, A., et al., A Stopped-Flow Light Scattering Methodology for Assessing the Osmotic Water Permeability of Whole Sertoli Cells, in Sertoli Cells. 2018, Springer, p. 279-286.
18. Campos, E., et al., 2011, Lack of Aquaporin 3 in bovine erythrocyte membranes correlates with low glycerol permeation. Biochemical and biophysical research communications, 408(3): p. 477-481.
19. Rodríguez, A., et al., 2014, Reduced hepatic aquaporin-9 and glycerol permeability are related to insulin resistance in non-alcoholic fatty liver disease. International journal of obesity, 38(9): p. 1213-1220.
20. Calamita, G., et al., 2012, Biophysical assessment of aquaporin-9 as principal facilitative pathway in mouse liver import of glucogenetic glycerol. Biology of the Cell, 104(6): p. 342-351.
21. Yang, B., T. Ma, and A. Verkman, 2001, Erythrocyte water permeability and renal function in double knockout mice lacking aquaporin-1 and aquaporin-3. Journal of Biological Chemistry, 276(1): p. 624-628.
22. Sakai, H., et al., 2014, Distribution of aquaporin genes and selection of individual reference genes for quantitative real-time RT-PCR analysis in multiple tissues of the mouse. Canadian journal of physiology and pharmacology, 92(9): p. 789-796.
23. Hess, R.A., et al., 1997, A role for oestrogens in the male reproductive system. Nature, 390(6659): p. 509-512.
24. Cooke, P.S., et al., 2017, Estrogens in male physiology. Physiological reviews, 97(3): p. 995-1043.
25. Fisher, J.S., et al., 1998, Immunoexpression of aquaporin-1 in the efferent ducts of the rat and marmoset monkey during development, its modulation by estrogens, and its possible role in fluid resorption. Endocrinology, 139(9): p. 3935-3945.
26. Pastor-Soler, N., et al., 2002, Expression of aquaporin 9 in the adult rat epididymal epithelium is modulated by androgens. Biology of Reproduction, 66(6): p. 1716-1722.
27. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.

28. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and cellular biochemistry, 408(1-2): p. 47-54.
29. Lebeck, J., et al., 2011, Estrogen prevents increased hepatic aquaporin-9 expression and glycerol uptake during starvation. American journal of physiology. Gastrointestinal and liver physiology, 302(3): p. G365-G374.
30. Oliveira, C.A., et al., 2005, Aquaporin-1 and− 9 are differentially regulated by oestrogen in the efferent ductule epithelium and initial segment of the epididymis. Biology of the Cell, 97(6): p. 385-395.
31. Hermo, L., et al., 2004, Cell specificity of aquaporins 0, 3, and 10 expressed in the testis, efferent ducts, and epididymis of adult rats. Journal of Andrology, 25: p. 494-505.
32. Ishibashi, K., 2009, New members of mammalian aquaporins: AQP10–AQP12, in Aquaporins. *Springer*. p. 251-262.
33. Madeira, A., et al., 2014, Human aquaporin-11 is a water and glycerol channel and localizes in the vicinity of lipid droplets in human adipocytes. Obesity. 22(9): p. 2010-2017.
34. Hermo, L., et al., 2008, Membrane domain specificity in the spatial distribution of aquaporins 5, 7, 9, and 11 in efferent ducts and epididymis of rats. The journal of histochemistry and cytochemistry, 56(12): p.1121-1135.
35. Yeung, C.-H.; Cooper, T., 2010, Aquaporin AQP11 in the testis: molecular identity and association with the processing of residual cytoplasm of elongated spermatids. Reproduction, 139(1): p. 209-216.
36. Eng, F., J.P. Wiebe, and L.H. Alima, 1994, Long-term alterations in the permeability of the blood-testis barrier following a single intratesticular injection of dilute aqueous glycerol. Journal of andrology, 15(4): p. 311-317.
37. Chiadak, J.D., et al., 2017, Lipopolysaccharide Modifies Glycerol Permeability and Metabolism in 3T3-L1 Adipocytes. International Journal of Molecular Sciences 18(12): p. E2566.
38. Tsukaguchi, H., et al., 1999, Functional and molecular characterization of the human neutral solute channel aquaporin-9. American Journal of Physiology 277(5): p. F685-F696.
39. Chua, M., et al., 2013, Revisiting oestrogen antagonists (clomiphene or tamoxifen) as medical empiric therapy for idiopathic male infertility: a meta-analysis. Andrology, 1(5): p. 749-757.
40. Jungwirth, A., et al., 2012, European Association of Urology Guidelines on Male Infertility: The 2012 Update. European Urology, 62(2): p. 324-332.
41. Kasturi, S.S., J. Tannir, and R.E. Brannigan, 2008, The metabolic syndrome and male infertility. Journal of andrology, 29(3): p. 251-259.

42. Schneider, G., et al., 1979, Increased estrogen production in obese men. The Journal of clinical endocrinology and metabolism, 48(4): p. 633-638.
43. Calamita, G., J. Perret, and C. Delporte, 2018, Aquaglyceroporins: drug targets for metabolic diseases? Frontiers in physiology, 9: p. 851.
44. Madeira, A., T.F. Moura, and G. Soveral, 2015, Aquaglyceroporins: implications in adipose biology and obesity. Cellular and molecular life sciences, 72(4): p. 759-771.
45. Martins, A.D., et al., 2015, Leptin modulates human Sertoli cells acetate production and glycolytic profile: a novel mechanism of obesity-induced male infertility? Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1852(9): p. 1824-1832.
46. Palmer, N.O., et al., 2012, Impact of obesity on male fertility, sperm function and molecular composition. Spermatogenesis, 2(4): p. 253-263.
47. Hofny, E.R., et al., 2010, Semen parameters and hormonal profile in obese fertile and infertile males. Fertility and sterility, 94(2): p. 581-584.
48. Rato, L., et al., 2016, New insights on hormones and factors that modulate Sertoli cell metabolism. Histology and histopathology, 31(5): p. 499-513.
49. Rojek, A.M., et al., 2007, Defective glycerol metabolism in aquaporin 9 (AQP9) knockout mice. Proceedings of the National Academy of Sciences of the United States of America, 104(9): p. 3609-3614.
50. Nicchia, G.P., et al., 2001, Tissue distribution and membrane localization of aquaporin-9 water channel: evidence for sex-linked differences in liver. The journal of histochemistry and cytochemistry, 49(12): p. 1547-1556.

Chapter 9

Expression of estrogen receptors alpha (ER-α), beta (ER-β) and G protein-coupled receptor 30 (GPR30) in testicular tissue of men with Klinefelter syndrome

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Expression of estrogen receptors alpha (ER-α), beta (ER-β) and G protein-coupled receptor 30 (GPR30) in testicular tissue of men with Klinefelter syndrome

Abstract

Klinefelter syndrome (KS) men present severe hormonal deregulation, particularly elevated serum estradiol concentration. Estrogens act through specific receptors and regulate testes development and spermatogenesis. Herein, we evaluated GPR30, ERα and ERβ mRNA expression in testis of KS men and men with 46XY karyotype and conserved spermatogenesis by reverse transcriptase and quantitative PCR. ERβ transcripts are the most abundant in testicular tissue of 46XY men. Notably, testicular GPR30 transcripts in KS men was approximately twelve times higher. Since GPR30 is essential to mediate estrogen effects over steroidogenesis, our data illustrates that it may underpin the testicular alterations observed in KS men.

Keywords: Estrogen receptors; Klinefelter syndrome; GPR30; Estrogen receptors alpha (ERα) and beta (ERβ), Spermatogenesis.

Introduction

Klinefelter syndrome (KS) is the most common sex chromosome abnormality in men. It is characterized by X chromosome polysomy, with disomy being the most common variant (47,XXY). Adults with KS present small testes, gynaecomastia and hormonal deregulation characterized by hypergonadotropic hypogonadism and altered sex steroid hormone levels. In fact, serum estradiol (E2) levels are high at the beginning of puberty and remain high throughout the onset of the adult life [1]. Estrogenic actions are mostly mediated by the classical nuclear estrogen receptors alpha (ERα) and estrogen receptors beta (ERβ). However, a G protein-coupled estrogen receptor (GPR30) was recently described in human testis [2]. Yet, E2 receptors expression in testis of KS men remains unknown despite the recognized alterations on E2 serum levels in these men. In this short communication we propose to unveil the alterations on mRNA expression levels of E2 receptors in testicular tissue of KS men.

Material and Methods

Patient's characterization

The clinical study of patients and testicle tissue processing were performed at the Centre for Reproductive Genetics Prof. Alberto Barros (Porto, Portugal). The study followed the Guidelines of the Local, National and European Ethical Committees and the Declaration of Helsinki. Only tissue samples left in culture plates after fertility treatment were used. Testicular biopsies were obtained from patients seeking for fertility treatment, after informed written consent. Six testicular biopsies were obtained from men with confirmed 46XY karyotype (age 44 ± 4 years), conserved spermatogenesis and seeking fertility treatment due to vasectomy or traumatic section of the vas deferens (Control group). Six additional testicular biopsies were obtained from azoospermic men (age 34 ± 3 years) with homogeneous 47XXY karyotype (KS group). Body mass index (BMI) of individuals of both groups were not significantly distinct, with KS individuals presenting an average value of 27 ± 3 kg/m^2 and control individuals a value of 29 ± 2 kg/m^2. Plasma hormone levels of individuals from Control group were 5.8 ± 0.5 IU/l for FSH, 5.0 ± 0.7 IU/l for LH, 5.4 ± 0.4 ng/ml for total testosterone, and 7.5 ± 0.7 pg/ml for free testosterone, and from KS group were 29.9 ± 5.1 IU/l for FSH, 5.2 ± 0.7 IU/l for LH, 3.3 ± 0.3 ng/ml for total testosterone, and 9.4 ± 0.3 pg/ml for free testosterone (Table 9.1). Testicular volume of individuals from KS group were 6.0 ± 0.5 ml and from Control group were 19.0 ± 1.5 ml. Individuals from both groups were not under any medication for at least 6 months at the time of sample collection (Table 9.1).

Table 9.1 Age, body mass index (BMI), mean testicular volume, and hormonal levels (follicle stimulating hormone (FSH), and luteinizing hormone (LH), total and free testosterone) of control and Klinefelter (KS) patients. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to control (P<0.05).

	Control Group	**KS Group**
Age (years)	44 ± 4	34 ± 3
BMI (kg/m^2)	25.7 ± 2.1	25.9 ± 3.6
FSH (IU/l)	5.8 ± 0.5	29.9 ± 5.1*
LH (IU/l)	5.0 ± 0.7	5.2 ± 0.7
Total testosterone (ng/ml)	5.4 ± 0.4	3.3 ± 0.3*
Free testosterone (pg/ml)	7.5 ± 0.7	9.4 ± 0.3*
Mean testicular volume (ml)	19.0 ± 1.5	6.0 ± 0.5*

RT_PCR and RTqPCR

Extraction of total RNA (RNAt) was performed using the EZNA Total RNA Kit (Omega Biotek, Norcross, USA). RNAt was reversely transcribed. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to analyse GPR30 (Sense: GGGGAGGGAGGTTTATTCT; Antisense: CGTGCACAGTCCCGC), ERα (Sense: GCCAGGCTTTGTGGATTTGA; Antisense: GATGTAGCCAGCAGCATGTC) and ERβ (Sense: AAGTTGGCCGACAAG; Antisense: ACAGGCTGAGCTCCA) mRNA expression as described [2]. Optimal annealing temperature (52ºC for GPR30; 60ºC for ERα and 62ºC for ERβ) and 35 cycles were used for amplification. Liver and heart cDNAs were used as positive controls for ERα and GPR30, and ERβ, respectively, and GAPDH was used as housekeeping gene (Sense: CGCCAG CCGAGCCACATC; Antisense: CGCCCAATACGACCAAATCCG; 60ºC, 35 cycles). Quantitative PCR (RTqPCR) was performed to evaluate GPR30, ERα and ERβ mRNA expression in testes as described [2]. Specific primers were designed for the amplification of the target and housekeeping transcripts (the same that were used in RT-PCR). Amplification conditions comprised an initial denaturation step of 5 min at 95ºC, followed by 35 runs of a 3 steps cycle: (1) a denaturation step of 10 s at 95ºC, (2) an annealing step of 30 s with a specific temperature for each set of primers and (3) an extension step of 10 s at 72ºC. Fold variation of the expression levels was calculated following the mathematical model proposed by Pfaffl.

Statistical Analysis

Experimental data are shown as mean ± SEM (n=6 for each condition in triplicate). Statistical analysis was performed using a one-way ANOVA in GraphPad Prism 6 (GraphPad Software, San Diego, USA). $P<0.05$ was considered significant.

Results

We detected the presence of ERα, ERβ and GPR30 transcripts in testicular tissue of men from Control and KS groups (Figure 9.1a). The relative abundance of all estrogens receptors mRNA transcripts was determined. ERβ transcripts were the most abundant (0.18 ± 0.07 arbitrary units) in testicular tissue of individuals from the Control group (Figure 9.1b), being its levels higher than those of GPR30 (0.026 ± 0.007 arbitrary units), the least abundant transcript. Notably, mRNA levels of GPR30 in testes of KS men were increased 12-fold when compared with men from the control group (0.32 ± 0.11 and 0.026 ± 0.007 arbitrary units, respectively). Interestingly, ERα and ERβ mRNA levels in testes of men with KS were not significantly altered when compared with testis from men of the Control group (ERα: 0.032 ± 0.008 and 0.051 ±

0.018 arbitrary units; ERβ: 0.063 ± 0.050 and 0.178±0.068 arbitrary units, respectively) (Figure 9.1b).

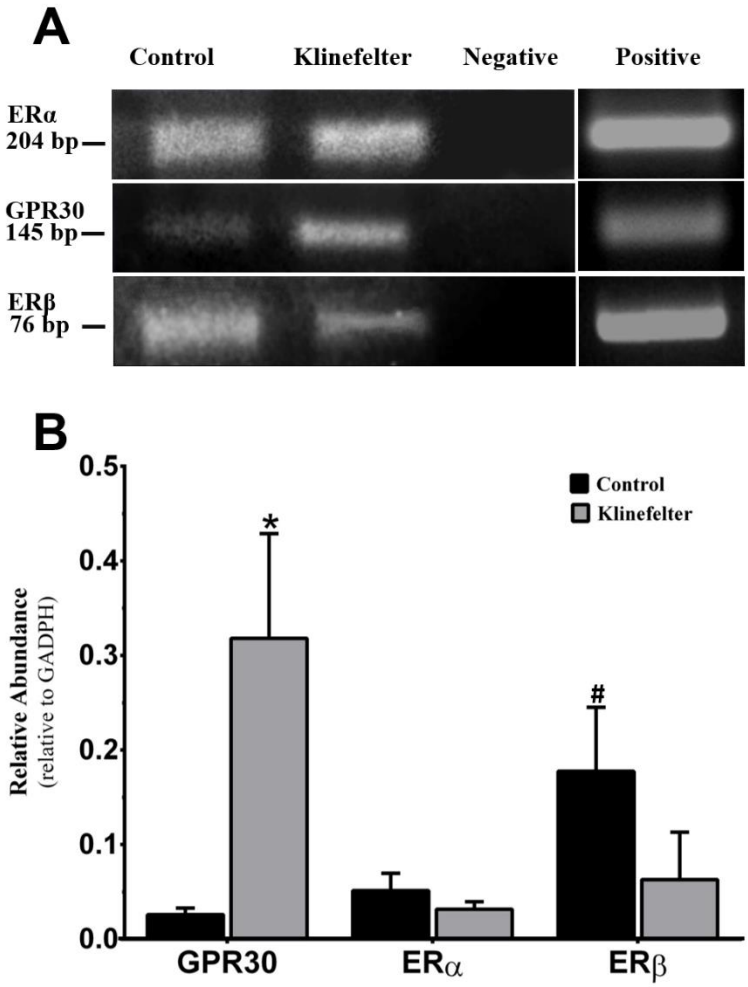

Figure 9.1 Identification and expression of estrogen receptors in human testicular tissue from Control men and men with Klinefelter syndrome. mRNA expression of G protein-coupled receptor 30 (GPR30), estrogen receptors alfa (ERα) and beta (ERβ). (**a**) Representative reverse transcriptase-PCR experiment. Liver cDNA was used as positive control for the evaluation of mRNA expression of ERα and GPR30, and heart cDNA for ERβ. (**b**) Relative abundance of GPR30, ERα and ERβ mRNA in testes of men from the Control and Klinefelter groups. Results are expressed as mean ± SEM (n=6). *Significantly different relative to Control group ($p<0.05$). #Significantly different to GPR30 group ($p<0.05$).

Discussion

Male fertility is a complex process targeted by endocrine factors. Within the seminiferous tubules, only Sertoli cells (SCs) possess receptors for testosterone. In testis, E2 is produced from androgens by aromatase, playing important roles in the development and physiology of

the male reproductive tract. High levels of E2 are present in the serum and intratesticular fluid of individuals with KS and also increased E2/testosterone ratios [3]. E2 alters SCs proliferation and animals treated with aromatase inhibitor or estrogen receptor antagonist present an increase in SCs numbers, illustrating that aromatase-driven E2 modulates SCs proliferative response [4].

E2 action is mediated by specific receptors (ERα, ERβ and GPR30), which present distinct pattern throughout the male reproductive tract. The classic nuclear ERs (ERα and ERβ) are present in testes, with ERα being expressed in interstitial cells, LC, spermatogonia, spermatocytes, round spermatids and spermatozoa, while ERβ is present in all testicular cell types with the exception of SCs and spermatogonia. In human testes, GPR30 is expressed in SCs, interstitial cells and diploid germ cells [2]. We detected GPR30, ERα and ERβ transcripts in human testicular tissue of KS and Control individuals. Notably, GPR30 testicular mRNA abundance in KS men is twelve times higher as compared with Control individuals. This may have a direct impact on testicular development (particularly on SCs proliferation) and physiology. Recent results [5] obtained from LC stimulation with E2 and GPR30 agonist, showed that activation of GPR30 lowers testicular testosterone levels. Our report is the first assessment of alterations on E2 receptors expression in testicular tissue of individuals with KS. Our results suggest that GPR30 may play an important role in testicular etiology of KS. Further studies are needed to unveil the physiological significance of this high GPR30 testicular expression in KS men since it can be regarded as a target to reduce E2 action and counteract the reproductive dysfunction in these men.

References

1. Wikström, A.M., et al., 2006, Are adolescent boys with Klinefelter syndrome androgen deficient? A longitudinal study of Finnish 47, XXY boys. Pediatric Research, 59(6): p. 854-859.
2. Oliveira, P.F., et al., 2014, Expression pattern of G protein-coupled receptor 30 in human seminiferous tubular cells. General and Comparative Endocrinology, 201: p. 16-20.
3. Hsueh, W., T. Hsu, and D. Federman, 1978, Endocrine features of Klinefelter's syndrome. Medicine, 57(5): p. 447-462.
4. Berger, T., et al., 2013, Increased testicular Sertoli cell population induced by an estrogen receptor antagonist. Molecular and Cellular Endocrinology, 366(1): p. 53-58.
5. Vaucher, L., et al., 2014, Activation of GPER-1 Estradiol Receptor Downregulates Production of Testosterone in Isolated Rat Leydig Cells and Adult Human Testis. PLoS ONE, 9(4): p. e92425.

Chapter 10

General Discussion and Conclusions

General discussion

Infertility and subfertility affect a significant proportion of the world population. The World Health Organization estimates that about 60 to 80 million couples currently suffer from infertility worldwide [1]. The cause for infertility may be due to problems only in the man, only in the woman or a result from alterations in both individuals. However, it has been highlighted that infertility affects about 7% of all men and 40 – 50% of infertility cases are due to the male factor [2, 3]. In addition, fertility rates among men younger than 30 years of age has decreased worldwide about 15% in the last decades [4]. Still, the causes for this phenomenon are not well known and about 30-45% of male infertility cases are classified as idiopathic [5]. In some of those cases, male infertility may be related to dysregulation in sex steroid hormones. It was previously reported that idiopathic infertile men have significantly higher levels of serum estrogens and estrogens/testosterone ratio than fertile men. However, decreased levels of estrogens in serum are also found in infertile men [6].

Hormonal production/regulation plays a crucial role in male reproduction. Hypotalamic – pituitary testis (HPT) axis constitute the main hormonal axis that controls male reproduction. The follicle stimulating hormone (FSH) is secreted by the pituitary in response to the secretion of gonadotropin releasing hormone (GnRH), which acts in testis, exclusively in the Sertoli cells (SCs) [7]. The testosterone that is produced by the Leydig cells in response to luteinizing hormone (LH), also acts on SCs through its specific receptors and androgen action in SCs is essential for a normal spermatogenesis [8]. Additionally, it was showed that estrogens also play an essential role in the establishment of male reproductive potential. In males, the most relevant biologically active estrogen is 17β-estradiol (E2) [9]. The primary source of E2 biosynthesis results from the aromatization of testosterone catalyzed by P450 aromatase [10]. E2 is produced in immature SCs, germ cells, Leydig cells, spermatozoa, in the epithelium of the efferent ductules and in the epididymal duct [11-15]. A high concentration of E2 was also reported in the testicular interstitial fluid, in the semen [8, 16] and in the *rete testis* [17]. Additionally, the concentration of E2 in the rat epididymis is about 25 times higher than in plasma [18], suggesting that it plays an essential role for the establishment of male reproductive potential. Indeed, it stimulates the renewal of spermatogonial stem cells, participates on the regulation of apoptosis and seminiferous tubules, as well as on efferent ducts homeodynamics [10]. In fact, it has been reported that E2, via estrogen receptors (ER), can modulate ion transport in the male reproductive tract and thus influence the ionic content and pH of the reproductive fluids [19-21]. Since hormonal dysregulation is frequently associated with male infertility [22] and the content of fluids that surround spermatozoa is essential for reproductive potential, it is of major relevance to investigate the possible

interconnection between hormonal dysregulation and ion transport in the male reproductive tract.

The homeodynamics of luminal fluids pH along the male reproductive tract is of extreme importance for the development, maturation, storage, capacitation and fertilizing ability of spermatozoa [23]. The production of seminiferous tubular fluid (STF), the fluid that surrounds all germ cells during spermatogenesis is mainly produced/controlled by SCs [24]. Among the several ions that constitute the tubular fluids in the male reproductive tract, HCO_3^- and H^+ have a major relevance, mainly because they have a pivotal role in the establishment and control of pH [25]. Hence, the maintenance of physiological pH in tissues, cells and fluids is dependent on HCO_3^- and CO_2 concentration [25]. Carbonic anhydrases (CAs) are metalloenzymes that catalyse the reversible hydration of CO_2 to H_2CO_3, that will be dissociated into HCO_3^- and H^+. They have a central participation in the regulation of ion, water and acid-base equilibrium in the body [26]. CAs are implicated in a net acid/base transepithelial transport in some epithelia and ducts, including epididymal ducts [27, 28]. So, in the first work we evaluated the role of HCO_3^- in human SCs (hSCs), through the general and selective inhibition of CAs. We identified three isoforms of CAs, one mitochondrial isoform (CA VB) and two cell membrane-bound isoforms (CA IX and CA XII) in hSCs. We further observed that HCO_3^- and its control by CAs is essential to hSCs once the inhibition of CAs with acetazolamide (general inhibitor) caused alterations in hSCs physiology. In those conditions, the nutritional support of spermatogenesis by hSCs is altered by a decreased production of HCO_3^-. Indeed, our data show that inhibition of CAs causes an increased production of lactate and alanine, without any alteration in glucose consumption, pyruvate production or intracellular LDH activity. Germ cells in development are unable to metabolize glucose and use the lactate produced by SCs as energy substrate [29, 30]. Interestingly, this work illustrates that diminished production of HCO_3^- decreases the expression of genes related to mitochondrial biogenesis, which may be due to the inhibition of the mitochondrial isoform (CA VB), as in the results attained with a selective inhibitor (SLC-0111) for CA IX and CA XII these alterations were not observed. Furthermore, CAs mitochondrial isoforms are implicated in lipogenesis [31]. When we inhibited CAs, we observed a significant alteration on hSCs lipid metabolism, particularly on the expression of hormone sensitive lipase (HSL), an enzyme responsible for the hydrolysis of triacylglycerol molecules. Although little is known about the significance of this specific pathway on SCs physiology, it was reported that it can serve as an intracellular source for the production of lactate under specific conditions [32, 33]. Hence, we have shown that CAs can play a key role in spermatogenesis by controlling hSCs metabolism and by modulating the expression of genes associated with mitochondrial biogenesis in hSCs.

Considering we seeing that HCO_3^- is essential for SCs and that HCO_3^- transporters are present in SCs, the formation of the STF by these cells may be compromised by alterations on the concentration of HCO_3^- [34-36]. Besides, SCs function is influenced by E2 levels. We aimed to evaluate the effects of high levels of E2 on the transport of HCO_3^- in hSCs. When these cells were exposed to elevated doses of E2 we observed an alteration in HCO_3^- transport, with a consequent alteration in the intracellular pH (pHi). We identified, for the first time the presence of the three HCO_3^- transporters of the solute carrier 4 (SLC4) family in isolated cultured hSCs. We identified the presence and cellular localization of the Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE), the electroneutral Na^+/HCO_3^- cotransporter 1 (NBCn1) and the electrogenic Na^+/HCO_3^- cotransporter (NBCe1). NDCBE is localized in the adluminal portion, while NBCn1 and NBCe1 are localized in the basal portion of hSCs plasma membrane. Protein expression levels of these three HCO_3^- transporters were increased in hSCs treated with elevated doses of E2, which resulted in increased pHi, due to increased HCO_3^- concentration in the intracellular compartment. We also observed that E2 alters the transcellular transport by SCs. E2-treated hSCs presented a reduced magnitude of the ATP-stimulated electrogenic transport of electrolytes, showing that ion transport through these proteins is compromised. Therefore, HCO_3^- transport is essential to hSCs physiology, and consequently to the production of STF, and its exposure to high doses of E2 causes an alteration in the transport of HCO_3^- leading to changes in pHi.

SCs ensure the BTB homeodynamics to establish a unique microenvironment in the adluminal compartment of the seminiferous epithelium [37]. This barrier controls the STF composition and the spermatogenesis through the establishment of a fully controlled environment with distinct characteristics of the extratubular fluids [23, 38]. SCs interactions involve dynamic and physical organization intermediated by distant stimuli, through the action of signalling molecules, growth factors and hormones [39, 40]. As reported in our studies and in many other previously published, E2 causes multiple alterations in the morphology, metabolism and ion homeodynamics of SCs [36, 41, 42], which can lead to changes in the establishment and maintenance of BTB with serious implications for spermatogenesis. It is known that the metabolism of triacylglycerol molecules is very important for SCs and that mice lacking mitochondrial glycerol phosphate dehydrogenase exhibit reduced fertility [43]. Moreover, increased concentration of glycerol in the testis causes sterility due to the destabilization of the tight-junctions between SCs [44, 45]. High glycerol concentrations are responsible for the disruption of the homeostasis in the tubular fluid tubular fluid homeostasis due to the impaired function of the BTB, leading to the apoptosis of germ cells. Interestingly, acute exposition to high concentration of glycerol leads to temporary arrest of spermatogenesis, while chronic exposition results in permanent oligospermia or even azoospermia [45, 46]. Therefore, due to

the great importance of glycerol in the testis, it is crucial to study the molecular dynamics of glycerol in BTB and how hormonal dysregulation can alter the events associated with it.

Among the several membrane transporters responsible for the STF composition, a new class has emerged in the last few years. Aquaglyceroporins (AQPs) are membrane transporters responsible for the membrane permeability to water and particularly to glycerol [47, 48], which include four isoforms: AQP3, AQP7, AQP9, and AQP10 [49]. Consequently, we evaluated the impact of high concentrations of E2 on mouse SCs (mSCs) AQPs expression, with focus on the expression of AQP3, AQP7 and AQP9 (the *Aqp10* gene is a pseudogene in mouse) and on mSCs glycerol permeability. Our results showed that mSCs express AQP9 and AQP3, but not AQP7, and AQP9 was the most abundant. High E2 levels decreased AQP9 expression and had no influence on AQP3 expression in mSCs. As AQPs are the main transporters of glycerol in physiological conditions, besides modulating the expression of AQP9, E2 also decreased the permeability of mSCs to glycerol. These data point towards the important physiologic role of E2 on mSCs glycerol transport, through modulation of AQP9. It is therefore evident that alterations caused by E2 in glycerol permeability may be related to fertility problems in males.

The establishment and maintenance of male fertility encompasses a series of complex events influenced by endocrine factors [50]. Klinefelter syndrome is a genetic reproductive disorder associated with hyperestrogenism. High levels of E2 were detected in both, serum and intratesticular fluid of individuals with Klinefelter syndrome [51, 52]. Increased E2/testosterone ratios, late increase in testosterone levels during puberty and increased circulating levels of E2 are also associated to Klinefelter syndrome [51]. Furthermore, adults with Klinefelter syndrome are characterized by elevated serum levels of FSH and LH [53], which may be due to decreased testosterone production [54], triggering a positive feedback on pituitary. This genetic disorder has a striking feature with a preponderant role in the spermatogenic dysfunction. Hyperestrogenism is associated to decreased proliferative potential of SCs (a condition that may be associated with hypogonadism observed in men with Klinefelter syndrome [55]). A positive linear correlation has also been found between testicular weight and the absolute number of SCs in several mammalian species [56]. Furthermore, hyperestrogenism is associated with testicular apoptosis, decreased germ cell numbers [57], decline on the expression of survival factors and an increase in apoptotic factors on cells of the seminiferous tubules [58]. E2 action is mediated by specific receptors: ERα, ERβ and GPR30. Our study in men with Klinefelter syndrome started by the evaluation of the expression of estrogen receptors in the testicular tissue. We detected a higher of GPR30 mRNA in the testicular tissue of men with KS. This increase may have a direct impact on testicular development (particularly on SCs proliferation) and physiology. In fact, recent data obtained

by Vaucher and collaborators [54], which stimulated Leydig cells with E2 and a GPR30 agonist, showed that activation of GPR30 lowers testosterone levels in testes. GPR30 seems to be an important mediator of E2 effects over steroidogenesis and may be partly responsible for the alterations observed in the testis of men with Klinefelter syndrome, namely the decreased testosterone levels and reduced gonadal weight. Our results suggest that GPR30 plays an important role in the infertility detected in men with Klinefelter syndrome and may be regarded as a possible target to reduce the action of E2 in these individuals.

Overall, our results expose new mechanisms by which E2 can regulate SCs physiology and consequently spermatogenesis. These mechanisms are essential to determine male reproductive potential and may explain male infertility associated with estrogen dysregulation.

References

1. Rutstein, S.O. and I.H. Shah, 2004, Infecundity infertility and childlessness in developing countries. Notas de Población, 14(41): p. 25-81.
2. Lotti, F. and M. Maggi, 2014, Ultrasound of the male genital tract in relation to male reproductive health. Human Reproduction Update, 21(1): p. 56-83.
3. Brugh, V.M. and L.I. Lipshultz, 2004, Male factor infertility: evaluation and management. Medical Clinics, 88(2): p. 367-385.
4. Martin, J.A., et al., 2007, Births: final data for 2005. National Vital Statistics Reports, 56(6): p. 1-103.
5. Jungwirth, A., et al., 2012, European Association of Urology guidelines on Male Infertility: the 2012 update. European Urology, 62(2): p. 324-332.
6. Andersson, A.-M., et al., 2004, Impaired Leydig cell function in infertile men: a study of 357 idiopathic infertile men and 318 proven fertile controls. The Journal of Clinical Endocrinology & Metabolism, 89(7): p. 3161-3167.
7. McLachlan, R., et al., 2002, Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Progress in Hormone Research, 57(1): p. 149-179.
8. Alves, M.G., et al., 2013, Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cellular and Molecular Life Sciences, 70(5): p. 777-793.
9. Baggett, B., et al., 1959, Conversion of C14-testosterone to C14-estrogenic steroids by endocrine tissues. Endocrinology, 64(4): p. 600-608.
10. O'donnell, L., et al., 2001, Estrogen and spermatogenesis. Endocrine Reviews, 22(3): p. 289-318.
11. Lambard, S., et al., 2004, Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors. Journal of Molecular Endocrinology, 32(1): p. 279-289.

12. Lambard, S., et al., 2003, Expression of aromatase in human ejaculated spermatozoa: a putative marker of motility. MHR: Basic science of reproductive medicine, 9(3): p. 117-124.
13. Carpino, A., F. Romeo, and V. Rago, 2004, Aromatase immunolocalization in human ductuli efferentes and proximal ductus epididymis. Journal of Anatomy, 204(3): p. 217-220.
14. Turner, K., et al., 2002, Development and validation of a new monoclonal antibody to mammalian aromatase. Journal of Endocrinology, 172(1): p. 21-30.
15. Foucault, P., M. Drosdowsky, and S. Carreau, 1994, Andrology: Germ cell and Sertoli cell interactions in human testis: evidence for stimulatory and inhibitory effects. Human Reproduction, 9(11): p. 2062-2068.
16. Purvis, K., et al., 1975, Indices of gonadal function in the human male: II. Seminal plasma levels of steroids in normal and pathological conditions. Clinical Endocrinology, 4(3): p. 247-258.
17. Free, M.J. and R.A. Jaffe, 1979, Collection of rete testis fluid from rats without previous efferent duct ligation. Biology of Reproduction, 20(2): p. 269-278.
18. Kumari, G., et al., 1980, Regional differences in steroidogenesis and hormone levels in the epididymis and vas deferens of adult rats. International Journal of Andrology, 3(1-6): p. 267-281.
19. Zhou, Q., et al., 2001, Estrogen action and male fertility: roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. Proceedings of the National Academy of Sciences, 98(24): p. 14132-14137.
20. Hess, R.A., 2000, Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Reviews of Reproduction, 5(2): p. 84-92.
21. Lee, K.-H., et al., 2001, Estrogen regulation of ion transporter messenger RNA levels in mouse efferent ductules are mediated differentially through estrogen receptor (ER) α and ERβ. Biology of Reproduction, 65(5): p. 1534-1541.
22. Hofny, E.R., et al., 2010, Semen parameters and hormonal profile in obese fertile and infertile males. Fertility and Sterility, 94(2): p. 581-584.
23. Pastor-Soler, N., C. Piétrement, and S. Breton, 2005, Role of acid/base transporters in the male reproductive tract and potential consequences of their malfunction. Physiology, 20(6): p. 417-428.
24. Mann, T. and C. Lutwak-Mann, Testis and testicular semen, in Male Reproductive Function and Semen. 1981, Springer. p. 83-138.
25. Swietach, P., R.D. Vaughan-Jones, and A.L. Harris, 2007, Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer and Metastasis Reviews, 26(2): p. 299-310.
26. Jacobs, M. and D.R. Stewart, 1942, The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. The Journal of general physiology, 25(4): p. 539-552.

27. Cohen, J.P., A.P. Hoffer, and S. Rosen, 1976, Carbonic anhydrase localization in the epididymis and testis of the rat: histochemical and biochemical analysis. Biology of Reproduction, 14(3): p. 339-346.
28. Caflisch, C.R. and T. DuBose Jr, 1990, Direct evaluation of acidification by rat testis and epididymis: role of carbonic anhydrase. American Journal of Physiology-Endocrinology and Metabolism, 258(1): p. E143-E150.
29. Oliveira, P.F., et al., 2015, The Warburg effect revisited—lesson from the Sertoli cell. Medicinal Research Reviews, 35(1): p. 126-151.
30. Dias, T.R., et al., 2013, Glucose transport and metabolism in sertoli cell: relevance for male fertility. Current Chemical Biology, 7(3): p. 282-293.
31. Scozzafava, A., C.T. Supuran, and F. Carta, 2013, Antiobesity carbonic anhydrase inhibitors: a literature and patent review. Expert Opinion on Therapeutic Patents, 23(6): p. 725-735.
32. Sauer, L., et al., 1980, Mitochondrial malic enzymes. Mitochondrial NAD (P)+-dependent malic enzyme activity and malate-dependent pyruvate formation are progression-linked in Morris hepatomas. Journal of Biological Chemistry, 255(9): p. 3844-3848.
33. Wise, E.M. and E.G. Ball, 1964, Malic enzyme and lipogenesis. Proceedings of the National Academy of Sciences, 52(5): p. 1255-1263.
34. Bernardino, R.L., et al., 2016, Estradiol modulates Na+-dependent HCO3− transporters altering intracellular pH and ion transport in human Sertoli cells: A role on male fertility? Biology of the Cell, 108(7): p. 179-188.
35. Bernardino, R.L., et al., 2013, Effect of prediabetes on membrane bicarbonate transporters in testis and epididymis. Journal of Membrane Biology, 246(12): p. 877-883.
36. Bernardino, R.L., et al., 2015, Estrogenic regulation of bicarbonate transporters from SLC4 family in rat Sertoli cells. Molecular and Cellular Biochemistry, 408(1-2): p. 47-54.
37. Gerber, J., J. Heinrich, and R. Brehm, 2016, Blood–testis barrier and Sertoli cell function: lessons from SCCx43KO mice. Reproduction, 151(2): p. R15-R27.
38. Bernardino, R.L., Formation and biochemistry of seminal plasma and male accessory fluids, in Andrology: Current and Future Developments, M.G. Alves and P.F. Oliveira, Editors. 2017, Bentham Science Publishers, p. 207-229.
39. Sharpe, R.M., et al., 2003, Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction, 125(6): p. 769-784.
40. Risley, M.S., I.P. Tan, and J. Farrell, 2002, Gap junctions with varied permeability properties establish cell-type specific communication pathways in the rat seminiferous epithelium. Biology of Reproduction, 67(3): p. 945-952.

41. Simoes, V., et al., 2013, Regulation of apoptotic signaling pathways by 5α-dihydrotestosterone and 17β-estradiol in immature rat Sertoli cells. The Journal of steroid biochemistry and molecular biology, 135: p. 15-23.
42. Alves, M.G., et al., 2012, In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1823(8): p. 1389-1394.
43. Lu, B., et al., 2008, A mutation in the inner mitochondrial membrane peptidase 2-like gene (Immp2l) affects mitochondrial function and impairs fertility in mice. Biology of Reproduction, 78(4): p. 601-610.
44. Eng, F., J.P. Wiebe, and L.H. Alima, 1994, Long-term alterations in the permeability of the blood-testis barrier following a single intratesticular injection of dilute aqueous glycerol. Journal of Andrology, 15(4): p. 311-317.
45. Wiebe, J.P., et al., 2000, Glycerol Disrupts Tight Junction—Associated Actin Microfilaments, Occludin, and Microtubules in Sertoli Cells. Journal of Andrology, 21(5): p. 625-635.
46. Wiebe, J.P., K.J. Barr, and K.D. Buckingham, 1989, Sustained azoospermia in squirrel monkey, Saimiri sciureus, resulting from a single intratesticular glycerol injection. Contraception, 39(4): p. 447-457.
47. Watanabe, S., et al., 2016, Aquaporin-9 facilitates membrane transport of hydrogen peroxide in mammalian cells. Biochemical and Biophysical Research Communications, 471(1): p. 191-7.
48. Agre, P., et al., 2002, Aquaporin water channels--from atomic structure to clinical medicine. Journal of Physiology, 542(Pt 1): p. 3-16.
49. Bernardino, R.L., et al., 2016, Hepatocyte and sertoli cell aquaporins, recent advances and research trends. International journal of molecular sciences, 17(7): p. E1096.
50. Flanagan, J. and M. Lehtihet, 2015, The Response to Gonadotropin-Releasing Hormone and hCG in Men with Prior Chronic Androgen Steroid Abuse and Clinical Hypogonadism. Hormone and Metabolic Research, 47(9): p. 668-73.
51. Hsueh, W., T. Hsu, and D. Federman, 1978, Endocrine features of Klinefelter's syndrome. Medicine, 57(5): p. 447-462.
52. Wikström, A.M., et al., 2006, Are adolescent boys with Klinefelter syndrome androgen deficient? A longitudinal study of Finnish 47, XXY boys. Pediatric Research, 59(6): p. 854-859.
53. Aksglaede, L., N.E. Skakkebaek, and A. Juul, 2008, Abnormal sex chromosome constitution and longitudinal growth: serum levels of insulin-like growth factor (IGF)-I, IGF binding protein-3, luteinizing hormone, and testosterone in 109 males with 47, XXY, 47,

XYY, or sex-determining region of the Y chromosome (SRY)-positive 46, XX karyotypes. Journal of Clinical Endocrinology and Metabolism, 93(1): p. 169-176.

54. Vaucher, L., et al., 2014, Activation of GPER-1 Estradiol Receptor Downregulates Production of Testosterone in Isolated Rat Leydig Cells and Adult Human Testis. PLoS ONE, 9(4): p. e92425.
55. Klinefelter Jr, H.F., E.C. Reifenstein Jr, and F. Albright Jr, 1942, Syndrome Characterized by Gynecomastia, Aspermatogenesis without A-Leydigism, and Increased Excretion of Follicle-Stimulating Hormone 1. Journal of Clinical Endocrinology and Metabolism, 2(11): p. 615-627.
56. Hess, R., et al., 1993, Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. Endocrinology, 132(6): p. 2607-2613.
57. Simoes, V., et al., 2013, Regulation of apoptotic signaling pathways by 5α-dihydrotestosterone and 17β-estradiol in immature rat sertoli cells. Journal of Steroid Biochemistry and Molecular Biology, 135: p. 15-23.
58. Correia, S., et al., 2014, Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility. Fertility and Sterility, 102(1): p. 299-306.

Conclusions

The maintenance of a proper metabolic and ionic content in the luminal fluids is essential for spermatogenesis, spermatozoa maturation and capacitation. Ion homeodynamic and pH control in physiological range are crucial for the maintenance of male reproductive potential. If the ionic and metabolic balance established throughout the male reproductive tract is disturbed, it can lead to alterations in spermatozoa production and physiology resulting in infertility/subfertility. This work highlights new findings concerning the crucial role of E2 in male reproductive tract. Hormonal dysregulation, particularly in the levels of E2, are described in several cases of idiopathic infertility and infertility related to genetic disorders (e.g: Klinefelter's syndrome). Although these syndromes are multifactorial pathologies, hyperestrogenism is a striking feature with a preponderant role on the spermatogenic dysfunction observed. In these cases, the possibility of treatment is reduced since the cause is unknown and the targets altered by the increase in E2 are unknown.

This work represents a first step in the understanding of how high levels of E2 can alter the STF homeostasis. It shows that changes in the testicular transport of HCO_3^-, H^+ and glycerol occurs as a consequence of high E2 levels that can be found in that tissue (Figure 10.1). As a consequence of these alterations in SCs and in the testis, the composition of the STF and the maintenance of spermatogenesis may be compromised. One of the pathological cases related with high levels of E2 and presenting severe alterations in male reproductive potential is Klinefelter's syndrome. In this study, we have detected that men with Klinefelter syndrome have marked alterations in the expression of estrogen receptors in the testis (Figure 10.1).

Overall, we report interesting and promising results that led to a better understanding of E2 role in modulating STF production. However, there is still much to be unveiled concerning the role of membrane transporters in male fertility, and particularly on the influence of E2 on those mechanisms. It would be of great interest to evaluate the expression and function of membrane transporters, in which we observed alterations after exposure to elevated E2 levels, in SCs and testicular tissue from individuals with hyperestrogenism, such as Klinefelter syndrome. However, the scarcity of samples has hampered this so far. Further, in spite of being a very ambitious study due to the extreme difficulty of the technique, it would be very interesting to evaluate the production and constitution of the tubular fluids along the male reproductive tract on the influence of hormonal dysregulation. Clearly, these are exciting topics that researchers working in the field of reproductive biology and membrane transport will be interested in the future and may aid much to the treatment of male infertility.

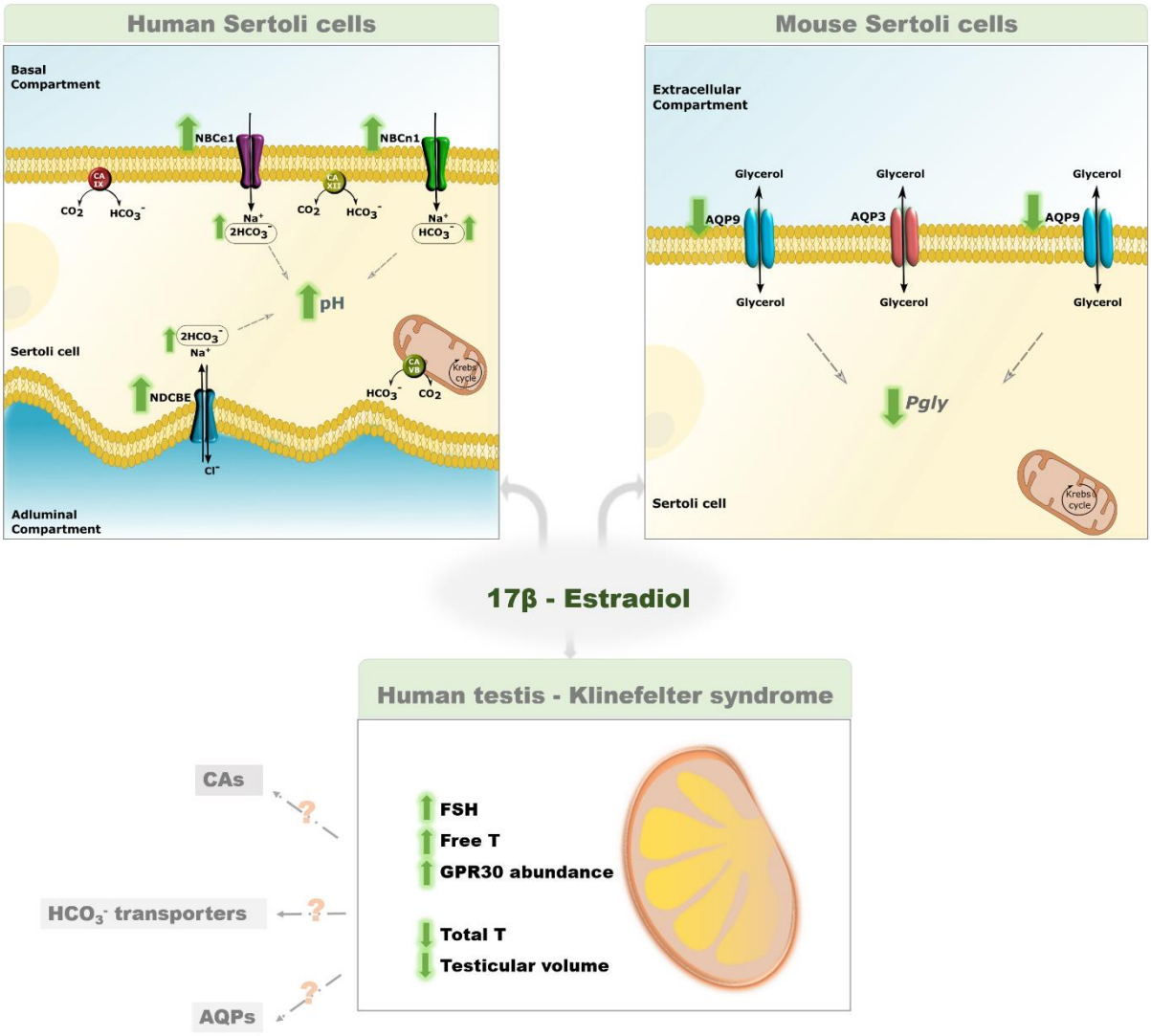

Figure 10.1. Effects of 17β – Estradiol (E2) on male reproductive tract. In human Sertoli cells (hSCs) three isoforms of carbonic anhydrases (CAs) were identified, CA VB, CA IX and CA XII, which are essential to HCO_3^- production. Three isoforms of HCO_3^- transporters were also identified in hSCs. Electrogenic Na^+/HCO_3^- cotransporters (NBCe1) and Electroneutral Na^+/HCO_3^- cotransporters (NBCn1) are localized in basal membrane, whereas Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE) is present in the adluminal membrane of hSCs. E2 is able to increase expression of NBCn1, NBCe1 and NDCBE, that results in increased concentration of HCO_3^- in intracellular compartment and consequently increased intracellular pH (pHi). In mouse SCs (mSCs), aquaglyceroporin 3 (AQP3) and the aquaglyceroporin 9 (AQP9) were identified, which participate on the transmembrane transport of glycerol. High levels of E2 cause a decrease in the abundance of AQP9 and consequently decreased permeability to glycerol (*Pgly*) in mSCs. Men with Klinefelter syndrome (KS) present hyperestrogenism. KS men show increased follicle stimulating hormone (FSH) and free testosterone (T) levels in plasma. The abundance of estrogen receptor GPR30 is increased in the testis of men with KS, whereas total T and testicular volume are decreased. Regarding the CAs, HCO_3^- transporters, and AQPs, nothing is known in the testis of men with KS, and further studies are needed to answer this question.

Annex I

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